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Chemical Markers of Transplantation Individuality Solubilized with Sonic Energy

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INTRODUCTION

Transplantation antigens are allotypic cell surface markers which stimulate an immune response by the host following grafting of foreign tissues. The specific accelerated rejection of repeat (second-set) tissue grafts derived from the original donor is the foundation of Medawar's hypothesis that transplants are destroyed by an immunologic reaction (86). Accelerated rejection signifies that the experience with the first graft induced a durable response expressed as resistance to the survival of a second transplant derived from the same donor or from an individual of similar genetic constitution. Thus, allografting induces the host to recognize a set of cellular antigens which distinguish him from the graft donor. These antigens constitute an allotypic system, i.e., a group of polymorphic specificities distributed among members of the same species. The recognition phenomenon expressed as transplantation immunity provides a sensitive tool to detect specific transplantation antigenic markers in tissues and subcellular materials (61).

Observations on the fate of normal tissue and tumor grafts led Little and later Snell, using murine lines, and Loeb, using guinea pig families, to suggest that graft survival is determined by the genetic relation between donor and host (61). Counce et al. (22) classified histocompatibility genes as strong or weak according to the speed of allograft destruction. If tumor grafts were unable to grow progressively and if skin grafts were destroyed within 15 days, the genetic locus controlling the antigenic difference between donor and host was considered to be strong. In several species there is a single, complex strong locus controlling the rapid rejection of allografts: H-2 of mice, B locus of chickens, AgB locus of rats, C-LA locus of chimpanzees, and HL-A locus of man (17, 24). The HL-A and H-2 loci have been studied most extensively. These loci code into a mosaic of serologically distinct specificities. Investigators postulate that the HL-A region is composed of multiple subloci. Each sublocus obeys the pattern of simple Mendelian inheritance of alleleic factors (24). The first sublocus contains the alleleic specificities HL-A 1, 2, 3, 9, 10, 11, and the second major sublocus contains the factors HL-A5, 7, 8, 12, 13. Batchelor and Selwood (8) stress that although the allelic relation of the determinants grouped in the first sublocus series suggests a distinct cluster of antigenic specificities, the sublocus concept is inconclusive at the present time. This concept fails to explain (i) the inheritance of other, "nonprivate", widely distributed, broad antigenic specificities (determinants 4a, 4b, 6a, 6b) and (ii) the production of alloantibody directed against specificities either detected on the recipient's cells or absent from the immunizing cell. Alternate patterns of inheritance have been reviewed by Amos (4). Bailey's (7) interesting

compilation of exceptions to the simple laws of transplantation, and the possible contribution of hybrid antigens, as discussed by Fox (34) must also be accounted for in any genetic theory of the histocompatibility loci. Indeed, many workers await further clarification of the chemical structure of the gene product transplantation antigens for clues to the translation mechanism. In spite of these theoretic uncertainties, a correlation appears between HL-A phenotypes and tissue graft survival. An appropriate match of the determinants detected in the HL-A mosaic of donor and host provides a higher likelihood of transplant success, and HL-A "typing" offers the brightest prospect for improved survival of transplants.

The weak histocompatibility loci, which may number 30 to 100 genetic sites in mice (46), control the prolonged graft rejection phenomena including the "rapid chronic" rejection at 19 to 61 days, "intermediate chronic" at 78 to 261 days, and the "prolonged chronic" at 307 to 550 days. Phylogenetically, chronic rejection is a primitive mechanism, for this pattern occurs frequently in amphibians. Of considerable significance are the additive or augmentative effects of weak histocompatibility loci, leading to the rapid destruction of grafts, as initially demonstrated by McKhann (79) and recently expanded by Graff et al. (41). Indeed, Eichwald and Weissman (31) showed that multiple non-H-2 differences in mice could mimic an H-2 (strong) difference; in both cases grafts were rejected within 14 days.

Klein investigated the difference between the expression of strong loci, which control rapid rejection phenomena, and weak loci, which control chronic graft rejection (68). He used H-2 differences which were restricted to incompatibility for a single antigenic factor. Some of the single incompatibilities resulted in chronic rejection of skin grafts, poor stimulation of humoral antibody formation, and cytologic changes in draining nodes more suggestive of rejection controlled by a non-H-2 (weak) rather than an H-2 factor. Klein proposed that each of the antigenic determinants within the H-2 locus initiated a unit response and that the greater immunogenicity of the strong antigenic difference was due to the multiple determinants included within a single complex locus. However, two observations suggest a qualitative difference between the immunological barriers determined by H-2 and by non-H-2 histocompatibility loci, even when both barriers result in the same graft survival times. First, McKhann (80) demonstrated that H-2 antigens are radioresistant, whereas non-H-2 antigens are radiosensitive. Second, Hilgert (47) showed that hosts which received allogeneic cells bearing a non-H-2 difference became tolerant to donor tissues, whereas those receiving cells possessing a monodeterminant H-2 difference developed immunity.

Although the weak histocompatibility antigens are present in cytoplasmic fractions (80), data derived from studies employing (i) immunofluorescent alloantibody, (ii) serologic absorption methods, and (iii) refined membrane preparations all suggest that the strong antigens reside on cell membrane surfaces (61). The antigenic information appears to be organized in "hot spots" on the cell surfaces. Boyse et al. (12) found the surface membrane to be studded with single clusters bearing H-2, Ly-A, Ly-B, and TL specificities. Davis and Silverman (26) saw clusters of $H\text{-}2^b$ and $H\text{-}2^d$ antigenic determinants on lymphocyte membranes with ferritin-labeled antibody. R. Ceppellini and B. Pernis (*personal communication*) localized immunofluorescent anti-HL-A 2 alloantibody TO11.03 at specific sites on the cell surface. Furthermore, antibody-blocking tests have demonstrated that after an antigenic specificity has been bound with alloantibody there is steric hindrance to the fixation of antibody molecules directed against other strong antigenic determinants, suggesting that the determinants are grouped together on the cell membrane.

The role of histocompatibility substances is almost certainly *not* to induce the artificial state of transplantation immunity. Theories concerning the physiological significance of transplantation antigens are based upon their strategic location on the cell surface and their strong immunogenicity. The concept of immunosurveillance, as initially proposed by Thomas (119) and later developed by Burnet (16), suggests that adaptive immunity developed to alert the host to the presence of tumor cells. Two types of observations support this hypothesis: (i) the increased incidence of neoplasia associated with immune deficiency diseases, with thymectomy, and with immunosuppression, and (ii) the high incidence of tumors in early life and in old age, periods when the immune response is believed to be less alert. The theory proposes that histocompatibility antigens serve as surface receptors which allow the immune system to distinguish new cells bearing autologous antigenic determinants from those bearing foreign determinants.

Mason and Warner (85) suggest that the lymphocyte surface carries immunoglobulin receptors. These workers reacted antiimmunoglobulin sera with lymphoid cells, mediating graft-versus-host reactions and adoptive transfer of delayed-type hypersensitivity (DTH). Cells treated with antilight-chain antibody had impaired reactivity. Mason and Warner thereby suggest that light chain is involved in the antigen recognition site on cells mediating DTH and transplantation im-

munity. Although they could not detect a heavy-chain component with their antiglobulin, they could not exclude the presence of a heavy chain belonging to a new immunoglobulin class. According to their hypothesis, lymphocyte messengers bearing immunoglobulin determinants conformationally homologous to transplantation antigens inform the individual of the array of cell-surface receptors present in his body.

In addition to this cell-to-cell mechanism, there might be a fraction of free histocompatibility antigen, either less tightly bound and dissociable from the cell surface or resulting from cell death. This material would perfuse regional lymph nodes in a fashion similar to the tissue-coding factors and mitotic control proteins postulated by Burch and Burwell (15). These telegraphic messages would assist in immunosurveillance and growth regulation.

The determinants of histocompatibility are intimately related to the factors controlling immune responsiveness. There are significant differences in the immune responsiveness of various mouse strains to an array of antigens (33). When different immune reactivities are selectively bred, they are found to be controlled by a small number of genes; in the case of synthetic amino acid polymers, they are controlled by a single autosomal gene (77) linked to the H-2 locus (78). Vaz and Levine (126) reported that although the same magnitude of immune response was induced in a variety of strains by a single large dose of antigen, variable responses were obtained with repeated small doses. These differences may be due to a varying affinity of the antigen receptors or to impaired responsiveness when the material is presented with restricted heterogeneity inherent in small doses. There was a correlation between the responsiveness of certain strains to hapten conjugates of bovine gamma globulin and of ovomucoid of hens and the responses of these same strains to synthetic amino acid polymers, suggesting that antibody production to a range of antigens might be determined at a single locus. Comparably, Gasser (35) found that a single recessive gene, controlling antibody synthesis against erythrocyte antigens, was linked to the weak histocompatibility loci H-3, H-6, and H-13.

A theory accounting for the relationship between genes coding for histocompatibility and for immune responsiveness has been postulated by Jerne (49). The germ line contains structural genes which code into antibody molecules specifically directed against histocompatibility antigens. Variable cistronic regions within these genes mutate yielding antibody molecules directed against antigenic specificities other than histocompatibility. Thereafter, the nonmutant, self-recognizing clones which are producing antibody against the host's own transplantation antigenic specificities are suppressed, leaving only those cells recognizing foreign specificities. Each individual possesses only a fraction of the mutant antibody diversity possible and thus has a different range of antibody specificity. This hypothesis can explain the large number of reactive lymphocytes which undergo blast transformation when confronted with foreign histocompatibility antigens in vitro, as well as the importance of histocompatibility loci in the development of immune responsiveness.

The sensitivity of lymphocytes to foreign transplantation antigens might be an important effector mechanism to destroy unwanted mutants by the immunosurveillance system, or an expression of a basic immunologic reactivity. Although this reactivity could also be explained by an anticlonal theory that lymphocytes are multipotential or by a hypothesis that the high density and avidity of histocompatibility determinants on intact cells can activate even low-affinity immunocompetent cells, Lawrence (73) has convincingly suggested that allotypic transplantation specificities are related to infectious agents. The large number of reactive cells in the nonsensitized host is due to prior antigenic exposure to cross-reacting materials in the environment. In accord are the observations of Rapaport and Chase (97) that streptococcal antigens possess cross-reactive antigenic determinants with rodent skin grafts and those of Hirata and Terasaki (48) that M protein extracts derived from streptococci inhibit alloantisera directed against all of the HL-A antigenic determinants. Allotypic blood group substances have been correlated with infectious agents. Crittenden et al. (23) showed that host susceptibility to subgroup B leukosis-sarcoma viruses was associated with an erythrocyte isoantigen which presumably acts as the the specific cell-membrane receptor substance required for infection. In a similar vein, Springer et al. (115) reported that the glycoprotein M and N blood group substances were related to the receptors for hemagglutination by influenza viruses. On the basis of optical rotatory dispersion studies, Jirgenson and Springer (50) concluded that these blood group substances and virus receptors contained alpha and beta conformations attributable to the peptide portions of these molecules. The biological properties of the molecules appeared to depend upon their peptide constituents because the disaggregation product (molecular weight 3×10^4) of the intact substance (molecular weight 12×10^6) had considerably less blood group and antiviral activity than the larger aggregates. Similarly, Lilly et al. (75) and Tennant and Snell (117) found that

susceptibility to leukemogenic viruses was qualitatively influenced by H-2 alleles. Thus, protective selective mechanisms probably operate to maintain alloantigenic polymorphisms in populations of animals infected with ever-changing viral phenotypes.

However, hypotheses attributing the function of transplantation antigens to the generation, maintenance, or expression (or to all three) of the immune response do not explain the reason why cells perpetuated in tissue culture retain their histocompatibility antigens (61). The persistence of transplantation antigens in vitro suggests that these materials have a primary role in the overall economy of the cell, either as part of the cytoarchitecture or in the mediation of crucial transport and contact phenomena. Thus, an antibody response directed against these substances inflicts damage tantamount to cell death. Elucidation of the chemistry and physiology of the histocompatibility antigens is expected to contribute to a better understanding of cell membrane structure and function.

EXTRACTION OF SOLUBLE TRANSPLANTATION ANTIGENS

Only during the last decade has the chemical nature of transplantation antigens come under intensive investigation, due to several serious strictures: (i) the limited numbers of available, genetically homogeneous animals; (ii) the cell membrane location of the materials, making them intractable in aqueous solvents; (iii) the denaturing effects of most techniques of solubilization; (iv) the low yields of antigen and the overwhelming array of contaminants; and (v) the imprecise and only crudely quantitative biological assays.

Although Medawar had postulated the existence of distinct transplantation antigens (86), many investigators believed that the expression of transplantation specificities required viable cells. Not until 15 years after Medawar's hypothesis were Billingham et al. (10) able to prepare an active subcellular fraction by exposing water-lysed murine cells to ultrasound. Antigenic material was found principally in membrane fractions, but it was present also in a soluble (supernatant) material. Although the initial investigations were primarily on the antigenic cell membrane fractions (33, 66, 81, 82), it rapidly became apparent that soluble materials had greater potential. First, water-soluble materials were amenable to an array of sophisticated fractionation techniques which facilitated purification and chemical characterization of the active principle. Second, Medawar (87) reported that the intravenous administration of crude semisoluble materials, particularly in combination with immunosuppressive agents, prolonged the survival of donor-specific tissue grafts. Thus, extraction of water-soluble transplantation antigen with sonic energy provided a powerful tool for chemical and biological studies of allograft immunity.

Criteria of Solubility

The concept of solubilization of transplantation antigens from cell membranes rested upon three assumptions: (i) biologically active materials could be isolated independent of membrane structure; (ii) the soluble substance represented the antigenic molecule in essentially the same form as on the cell surface; and (iii) there were no immunologically significant linkages between the soluble antigen and the residual insoluble material. Although transplantation determinants are more immunogenic when associated with cell membranes, biological studies demonstrated that soluble antigens could induce either accelerated rejection or prolonged graft survival, could elicit DTH reactions, and could inhibit specific tissue-typing alloantibodies.

Four criteria define soluble antigenic activity. Since only small amounts of material are generally obtained, solubility has not been defined with physical methods, e.g., solubility curves and light-scattering techniques. The most widely applied criterion has been the resistance of "solubilized" materials to sedimentation in an ultracentrifugal field. Early investigators considered antigens "soluble" if, after exhaustive dialysis in the absence of the solubilizing agent, they did not sediment at 105,000 $\times$ g. This criterion is unacceptable in view of two observations. First, Rapaport et al. (98) showed that materials which were soluble at 105,000 $\times$ g sedimented in 200,000 $\times$ g gravitational fields and contained fine membranous and vesicular structures under the electron microscope. Second, some membranous materials cannot sediment even in high-force fields due to the disaggregating nature of the suspending medium or to their low buoyant density (81, 82). Antigens solubilized by sound did not sediment at 200,000 $\times$ g, even after exhaustive dialysis against weakly ionic solutions or against distilled water. The solution in the base of the centrifuge tube after centrifugation at 200,000 $\times$ g did not contain membranous material upon ultrastructural analysis and possessed the same activity as solutions from other portions of the tube (43). Equilibrium density ultracentrifugations showed that the antigenic material had a buoyant density greater than 1.2, sedimenting in 1.5 M sucrose or 26% sodium bromide (53). Thus, if there was any lipid present it was extremely well masked by protein. Thus, by sedimentary charac-

teristics the sonically treated materials were "soluble."

The biological behavior of the antigen affords a second criterion. Solubilized transplantation antigens should be handled in the same fashion as other soluble antigens: rapid filtration and little retention after intravenous administration. Indeed, this very biological property is the basis of tolerogenicity. The inability of papain-treated antigenic materials, claimed to be soluble on the basis of 105,000 × *g* sedimentary characteristics, to elicit transplantation tolerance was probably related to their true biological (and chemical) "insolubility" (40).

The soluble state can be defined in a third, practical sense as follows: the antigen should be amenable to purification procedures in aqueous systems without appreciable losses of activity. For example, 60% of the total antigenic activity present in the crude extract solubilized with sound can be recovered as an electrophoretically homogeneous component, with marked increase in specific activity. On the other hand, only 2 to 5% of the activity of the crude fraction prepared by papain digestion of membrane fragments could be recovered in a more purified fraction (112). This impressive loss of activity must be related to a separation of papain-treated antigenic material from the aqueous phase, i.e., incomplete "solubilization."

The fourth and best criterion of solubilization rests upon the physical properties of the purified antigen. The purified electrophoretic component derived from crude, sonically treated suspensions is a protein of relatively low molecular weight with a well-defined chemical composition.

Solubilization with Sonic Treatment

Low-intensity sonic energy has been employed extensively in the extraction of intracellular and membrane-bound materials, e.g. components of the tricarboxylic acid cycle, membrane-bound enzyme systems, and labile cell constituents (for review, *see* 61). Sonic (below 16,000 cycles/sec) energy is gentler to biological activities than is ultrasonic (greater than 16,000 cycles/sec) energy. When cells or membranous preparations were exposed to ultrasonic energy of 20,000 cycles/sec, 60 w/cm^2 delivered by a probe apparatus, transplantation antigenic activity was rapidly lost, consistent with the effects of this energy in breaking covalent bonds and disrupting asymmetric molecules due to marked local heating effects and the generation of eddy currents (61). On the other hand, direct exposure of dissociated cells or isolated membranes to low intensity (9,000 to 10,000 cycles/sec, 15.5 w/cm^2) sonic energy liberated water-soluble materials with marked transplantation antigenic activity. Furthermore, sonic treatment of a solution containing either 2 to 3 mg of soluble antigen per ml or resuspended membranous precipitate did not diminish the biological potency of soluble or membranous antigenic determinants, suggesting that this type of sonic treatment does not denature the active moiety (101).

The conditions of sonic treatment must be closely regulated: (i) the frequency and intensity, (ii) the temperature, (iii) the cell concentration, and (iv) the exposure time. The effects of sonic treatment are determined by the frequency, and more importantly, by the intensity of the energy. The precise mechanism of antigen solubilization is unclear. Although sound can suspend insoluble substances (e.g., mercury), in water the conformance of the sonically treated antigens to the solubilization criteria enumerated above precludes the sonic effect from being a simple suspension of membranous material. The physical effects of sonic treatment include (i) cavitation due to rapid expansion and violent collapse of air bubbles entrapped in the liquid phase, (ii) mechanical actions including agitation analogous to foaming and friction from shearing owing to the transport of molecules at different rates than the solvent, and (iii) caloric generation due to local heating. Chemical effects include transpositions due to heat evolution and oxidations producing hydrogen peroxide, activated oxygen, and free radicals. At the intensity achieved in the system involving 9,000 to 10,000 cycles/sec, the chemical effects are less prominent than the physical effects. Therefore, solubilization of transplantation antigen probably represents a depolymerization of membrane antigen or a disruption of noncovalent forces between a basically soluble antigen and insoluble surface components.

The source of the sonic energy is a Raytheon model DF 101 magnetostrictive oscillator, which is primarily a radiofrequency-generating device. In accordance with Faraday's principle, a plate voltage generates oscillations of a nickel-laminated rod which is seated within a treatment stand coil-excitation field. The rod oscillations transduced as excursions (0.001 to 0.0015 inch) of a stellite diaphragm, generating sonic waves at a frequency of 9,000 to 10,000 cycles/sec. The 250-w input must be converted to at least 175-w radiofrequency output (70% efficiency). A voltmeter spanning the plate monitors its voltage during the sonic process; if the apparatus is malfunctioning, either insufficient voltage is generated or the voltage falls rapidly. The intensity of sonic treatment (i.e., the efficiency of the conversion of the radiofrequency to physical energy, which should be about 30%) can be measured with a cavitation

meter or by a caloric test. The adequate acoustic power (42 w) corresponds to a diaphragm area of 15.5 w/cm². In each of four instances when we investigated claims that the sonic-treatment method failed to liberate soluble antigen, the sonicators were malfunctioning. After appropriate technical servicing, these machines developed the proper power characteristics and yielded active substances.

Second, the temperature in the sonic-treatment chamber must be kept at 7 C or less by perfusion of the apparatus with a mixture of alcohol and water cooled to −10 C. Although localized heating does occur, particularly at interfaces, maintenance of a cool chamber prevents denaturation of the antigenic material, which is thermolabile above 50 C.

The third important parameter is the concentration of the cell suspension. Dense suspensions cannot be disintegrated because the energy dissipates as heat. On the other hand, dilute suspensions are subject to more pronounced sonic effects, resulting in excessive fragmentation and the release of a large amount of contaminants. Cell suspensions must be free of clumps because sonic energy breaks aggregates before it liberates antigen and because it is impossible to estimate the true cell content of clumped suspensions. Although the omission of divalent cations in the suspension medium or the treatment of suspensions with enzymatic agents which disperse cell clumps (e.g., collagenase or trypsin) might improve the uniformity of dispersion, these maneuvers appear to affect the antigenic activity adversely and to interfere with the sedimentation of contaminant intracellular organelles.

The fourth variable is the time of sonic exposure. A description of the effect of sonic energy on a cell population requires a three-dimensional plot of the antigenic activity versus the cell concentration versus the time of exposure. Whereas brief 2-min periods of exposure liberate activity from cell-free membrane preparations or from fetal cells (86), adult cells require periods of 5 to 7 min. Although prolonged sonic exposure has little detectable deleterious effect on the antigenic activity per se (Fig. 1), it fragments intracellular organelles, particularly nuclei, and liberates additional contaminants which may decrease the specific activity and interfere with purification. The point of maximal antigen release has been standardized by two methods, morphological and particle counting. (i) Morphologically, sonically treated suspensions with maximal activity show a disrupted surface membrane with an intact nucleus surrounded by a halo of ill-defined cytoplasm. (ii) After exposure to sonic energy, particle determinations with a Coulter counter set at

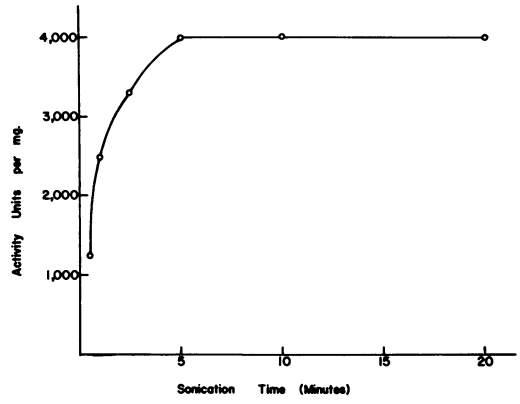

FIG. 1. *Effect of prolonged sonic treatment. The period of exposure of 40 × 10⁶ cultured peripheral lymphocytes per ml to 9,000 to 10,000 cycles/sec sonic energy was plotted against the inhibitory activity of the soluble antigen in the microcytotoxicity assay. Maximum yield occurred at 5 min. There was little detectable decline in antigenic activity after a 20-min exposure.* (*101*).

different size thresholds distinguish intact cells from subcellular residues. The point of maximal liberation occurs when 90% of the intact cells have been disrupted to smaller fragments; for human peripheral lymphocytes suspended at a concentration of 40 × 10⁶ cells per ml, this occurs at 5 min (Fig. 1).

Of considerable theoretical importance is the similarity of the antigenic material liberated by sonic treatment to that solubilized by treatment with 3 M KCl. In the latter method, 10^9 cells which have been washed twice with 0.9% KCl are admixed with 50 ml of 0.15 M NaCl containing 3 M KCl. The suspension is gently agitated at 4 C for about 18 hr and then centrifuged at 154,000 × *g* for 6 hr. Excess salt is removed by gel filtration on Sephadex G-25, and concentration is effected by dialysis against dry Sephadex or by partial lyophilization. An extraction period of 16 hr and a temperature of 4 C are optimal. Longer and repeated extractions of the sediment yield more antigen, but of lower specific activity. Extended extraction times decrease the amount of antigen recovered (101).

Source Material for Antigen Extraction

The sonic-treatment method has effectively solubilized transplantation antigens from all cells tested to date, including murine adult (53), human adult (64), and fetal (86) spleen, lung, liver, and kidney cells; canine spleen cells and peripheral lymphocytes (123); rat spleen cells (129); murine (43) and human cell membrane preparations; and guinea pig spleen, lung, liver, kidney (57, 59), and

sarcoma (56) cells. Although adequate quantities of material for biological and chemical characterization were obtained from inbred murine and guinea pig strains, there was no similar source of human material of uniform genetic constitution. Only limited amounts of material could be obtained from the organs of a single donor. Since the phenotype of each donor was different, comparison between extraction yields and experimental results with various preparations was impossible.

Through the efforts of George Moore and his colleagues (91), lines of peripheral lymphocytes derived from normal donors have been perpetuated in continuous cultures. These lymphoid lines possess a normal karyotype, contain all of the antigenic specificities present on the lymphocyte donor's peripheral cells, have a generation time of 20 hr, and have a maximal growth yield of 1.5×10^6 to 2.0×10^6 cells/ml. The cells perpetuate as large lymphoblasts which contain quantitatively more antigen per cell than do the donor's peripheral lymphocytes (Table 1), representing not only a vast but also a rich source of antigen. Although Moore and his colleagues cannot uniformly coax every individual's cells into continuous culture, other investigators have succeeded in doing so with phytohemagglutinin (14) or with cell-free lysates from patients with infectious mononucleosis and presumably carrying EB virus (18). Although it is possible that all established lymphoid cell lines represent viral transformants, neither EB nor any other viral particle has been detected in the lines derived from normal donors and initiated in the fashion described by Moore.

Cell lines derived from a normal donor and free of detectable infectious or malignant agents offer several advantages over lines derived from donors bearing malignant disease or carrying known viral agents. (i) There is less hazard of transmitting these agents to normal patients during biological studies and therapeutic applications of the antigenic materials. (ii) There is no contribution of viral or tumor specificities in the preparations. (iii) There tends to be less chemical complexity in extracts from normal tissue than from neoplastic material (56). (iv) Long-term continuous comparisons between the donor's peripheral lymphocytes, his cultured cells, and the purified antigen permit the integration of new knowledge into the study of the extracted material.

Problems heretofore unapproachable with inbred lines of animals or single human donor can be studied with continuous-cell cultures. Tissue cultures fed precursor amino acids use 1% of the total incorporated label to synthesize transplantation antigen. Microquantities of ^{14}C-labeled antigen can then be detected during analytical and purification procedures, thus overcoming the inefficiencies of radio-iodine labeling. In addition, pulse-labeling techniques yield information about the rate of biosynthesis and turn over of the antigen on the cell-surface membrane. With the techniques of cell fusion, it may become possible to generate mutant lines possessing special, more restricted HL-A mosaics useful for genetic and chemical studies on the expression of transplantation antigens.

TABLE 1. *Antigenic content of donor peripheral lymphocytes and of cultured cells from RPMI 1788 line*

Cell source	Inhibition in AD_{60} units		
	HL-A 2	HL-A 7	HL-A 5
Donor peripheral leukocytes[a]	3,000	3,500	12,000[b]
RPMI 1788 cells	850	1,100	30,000

[a] Contain approximately 50% granulocytes.
[b] End point not determined.

Fractionation of Soluble Antigens

Concentration of the crude material, which is obtained in dilute solution after sonic treatment, is the first step in the purification of the antigen. After ultracentrifugation of the sonically treated suspension, liters of crude supernatant antigen must be concentrated (i) by dialysis against hygroscopic materials, (ii) by ammonium sulfate precipitation, or (iii) by adsorption to diethylaminoethyl (DEAE)-cellulose resins, prior to fractionation.

Dialysis against Aquacide 1, a large-molecular-weight, branched, hygroscopic polysaccharide, achieved 15-fold concentration of crude antigen within 6 hr. However, there was a 30 to 40% loss of activity probably as a result of (i) rapid concentration with adsorption of protein to the dialysis tubing and denaturation by dehydration of the active moiety, (ii) possible *p*H changes in the sample, and (iii) the release of substances from the resin which irreversibly affected the antigen. When Sephadex G-200 was used as the hygroscopic substance, there was a slower concentration but only modest loss of activity.

Alternatively, crude guinea pig ultracentrifugal supernatant antigen was adsorbed onto DEAE-cellulose columns equilibrated with 0.05 M glycine, 0.02 M tris(hydroxymethyl)aminomethane (Tris), and 0.05% mannitol at *p*H 8.0. Two-thirds of the active principle was eluted with 0.5 M glycine, 0.2 M Tris, 0.5% mannitol, and 0.2 M NaCl at *p*H 8.0.

The other one-third could not be desorbed with buffers of high ionic strength (3.0) or at *p*H 10, suggesting either denaturation or a strong interaction between the antigen and the supporting medium. The nonspecific adsorption was slightly reduced with a Sephadex supporting medium; however, there were still considerable losses of total antigenic activity.

The most convenient method was ammonium sulfate fractionation. Serial exposures to 5, 10, 15, 25, 30, 50, and 78% ammonium sulfate saturations revealed that antigen precipitated in rather constant amounts between 30 and 50%. Above 50%, a large amount of nonspecific material was also sedimented, decreasing the specific activity. The ammonium sulfate fraction could be resolubilized in 0.9% NaCl, at 50 μg of protein per μliter. The concentrated solutions could then be applied to fractionation media in a thin layer. This precipitate possessed a 2.5-fold increase in ID_{50} value, a 10-fold increase in specificity ratio, and a 50-fold increase in avidity units over the crude, sonically treated HL-A antigen. (See below for explanation of serological terms.)

Fractionation of the crude antigen on Sephadex G-200 revealed three size classes in the 105,000 × *g* supernatant fluid of murine spleen cells. The active fraction was included within the gel (53). This early work in murine systems demonstrated that the gel must be properly swollen and that a sufficiently polar buffer must be used to minimize interactions between the antigen and the components excluded from the gel interior. Similarly, the active principle of guinea pig (57) and of human splenic (64) sonically treated transplantation antigens eluted in the included fraction (K_D 0.92) (Fig. 2). The antigenic activity of the Sephadex fraction was at best fivefold greater than that of the ammonium sulfate fraction. However, good separation of the antigenic fraction required up to 30-hr filtrations, and extracts from spleen, lung, liver, and kidney were sensitive to prolonged storage at 4 C in this crude state. (Materials from lymphoid cell lines, on the other hand, can tolerate 4 C for months.) Unfortunately, the organ extracts required Sephadex chromatography to separate the antigenic material from hemoglobin contamination, whereas the cultured cells did not.

The application of DEAE and CM-cellulose resins, in spite of appreciable losses of activity from nonspecific adsorption, yielded excellent separations of crude fractions possessing antigenic activity (57).

Figure 3 shows the reproducibility of the method. Three equal portions of a single batch of cultured human peripheral lymphocytes were exposed to sonic energy, sedimented at 135,000 × *g*, concentrated by 30% ammonium sulfate precipitation, and fractionated on Sephadex G-200. There was less than 10% difference in the activity of the triplicate ultracentrifugal supernatant fluids, the ammonium sulfate concentrates,

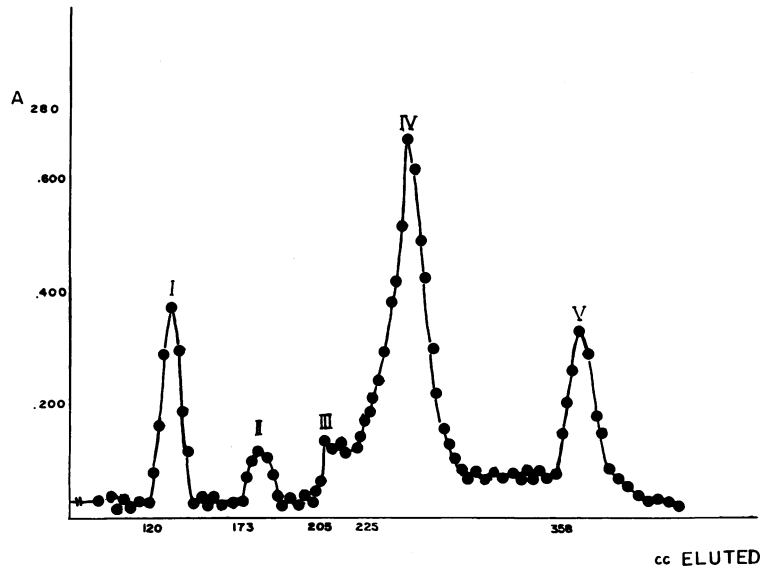

FIG. 2. *Elution pattern of 135,000 × g supernatant fluid passed through Sephadex G-200. Crude solubilized guinea pig antigen was charged onto a calibrated (2.5 by 90 cm) column of gel equilibrated with 0.5 M glycine, 0.2 M Tris, 0.5% mannitol at pH 8.0. There were five components in the included volume. Only fraction I, K_D 0.92, possessed antigenic activity (57).*

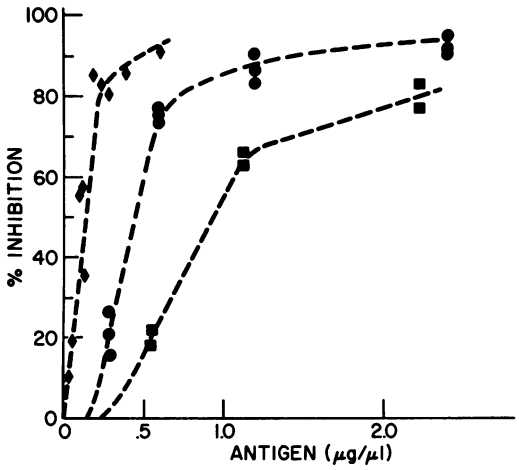

FIG. 3. *Reproducibility of the extraction and initial purification procedure. A large batch of cultured cells was divided into three equal samples. The cells were exposed to sound and then were ultracentrifuged, yielding supernatant antigen (■) which was then precipitated with 30% ammonium sulfate (●) and finally passed through Sephadex G-200 (◆) to yield the K_D 0.92 fraction I. There was less than a 10% difference between the degrees of inhibition observed with each of the triplicate samples after each maneuver (101).*

and the Sephadex G-200 fractions as inhibitors of immune cytolysis by a specific anti-HL-A 2 alloantiserum.

Purification on Polyacrylamide Gel Electrophoresis

The most powerful tool in the purification of transplantation antigenic activity is polyacrylamide gel electrophoresis (PAGE). The applications, instrumentation, and general strategy of PAGE were described by Chrambach and Rodbard (19). One of the advantages of these systems is the molecular stacking which occurs in the moving boundary between two buffer phases. This effect results in (i) concentration effect, facilitating the analysis of dilute samples; (ii) ultrathin starting zones, improving resolution; and (iii) capability for "steady-state stacking" conditions, permitting the application of large protein loads. A second advantage is the simultaneous discrimination of differences in molecular size and charge. Variable sieving of identically charged molecules can be accentuated by adjustment of the pore size, which is determined by the polyacrylamide concentration, or by the use of pore gradients.

Twenty distinct electrophoretic components were revealed by running the guinea pig Sephadex G-200 fraction 1 in a 7.5% gel at *p*H 9.4 (Fig. 4). Biological assays localized the guinea pig antigenic activity to a component with R_F 0.73 to 0.74 (Fig. 5). The component was isolated from lung, liver, kidney, and spleen of guinea pigs; in each case, it alone possessed the transplantation activity (Fig. 6). It was electrophoretically homogeneous in gels run at different *p*H values with various pore sizes and in the presence of 8 M urea (Fig. 7).

The active Sephadex fraction derived from human spleens (64) and the 30% ammonium sulfate precipitate derived from cultured peripheral lymphocytes (101) both yielded an active component with R_F 0.80. Purification of the splenic component was impaired by a contaminant with a very similar electrophoretic mobility, R_F 0.84, which could be removed by dissociation in 15% acrylamide gels containing 8 M urea. Fortunately, this contaminant protein was not present in the crude fractions from lymphoid cell lines.

To obtain sufficient material for extensive biological and chemical characterization, preparative acrylamide gel systems were essential. A proper translation from the analytic to the preparative scale has been restricted by the available PAGE equipment. For maximal resolution, protein loads cannot exceed a gel surface of 1 mg/cm², limiting the load to 10 to 15 mg of protein. Since solubilized extracts contained about 2% HL-A antigen, only microgram amounts of purified material could be obtained by a preparative run.

This tedious method has now been overcome by a more efficient approach based upon steady-state stacking principles. The upper and lower stacking limits were chosen to bracket the mobility of the HL-A antigen, yet be sufficiently narrow to exclude greater than 90% of the inactive contaminants from the stack. The contaminants migrated as a diffuse smear behind the stack. Selective preparative stacking of the active material yielded a prepurified fraction, constituting 10% of the crude material. The prepurified material was then charged onto a second preparative gel at 10 times the concentration of active antigen, since 90% of the contaminants had been removed. Thus, 1- to 2-mg batches of electrophoretically purified material were obtained from the PAGE system (Fig. 8, 9).

The efficiency of the second electrophoretic step was improved with isotachophoresis. A steady-state stacking system was run at low currents in a soft gel containing spacers of either synthetic ampholines with broad distributions of mobilities or specific ions with mobilities between that of the antigen and its adjacent contaminants. With this modification, up to 300-mg prepurified antigenic protein loads could be applied onto gels with surface areas of 10 cm². With further development, isotachophoresis should be able to accommodate gram loads of material.

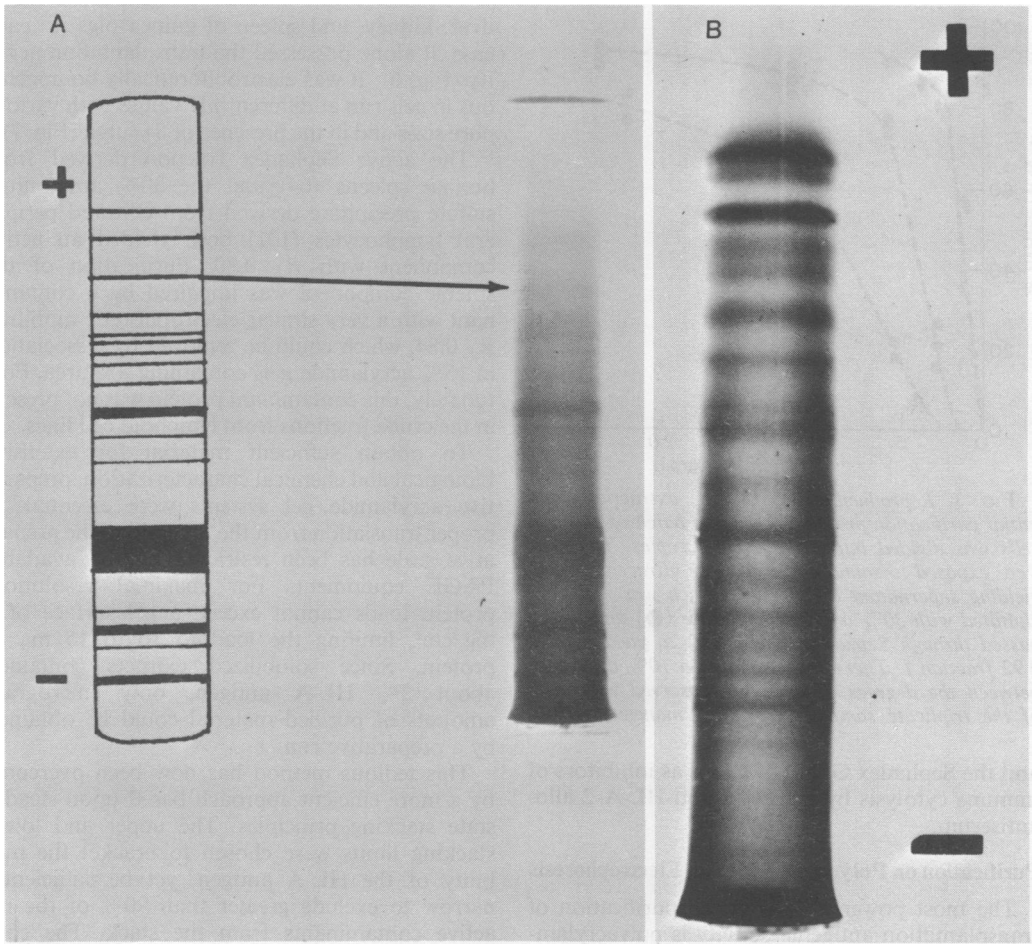

FIG. 4. *Heterogeneity of crude antigen fractions. Part A shows an acrylamide gel (7.5%, pH 9.4) after electrophoresis of Sephadex fraction I derived from sonically treated guinea pig spleen cells. A schematic diagram of the gel is shown on the light. The R_F 0.73 to 0.74 component, possessing biological activity, is indicated with an arrow (57). Part B shows an acrylamide gel following electrophoresis of a 30% ammonium sulfate precipitate derived from human cell line RPMI 1788. The R_F 0.80 component possessed biological activity (101).*

Recent advances in obtaining continuous lymphoid cell lines as a large, reproducible antigen source; in developing simple, reproducible extraction procedures; and in applying PAGE with appropriate stacking parameters have yielded large amounts of purified material for characterization of the transplantation antigens.

BIOLOGICAL ACTIVITIES OF SONICALLY SOLUBILIZED TRANSPLANTATION ANTIGENS

Transplantation antigenic activity can be detected by immune reactions that recognize the allotypic expression of these determinants. The most important immune response is allograft rejection. Medawar (86) defined transplantation antigens as materials that induce normal hosts to reject donor-type skin grafts in accelerated fashion (the second-set phenomenon). On the other hand, antigenic activity can be demonstrated by specific prolongation of donor graft survival. Whereas accelerated rejection requires that the extract contain but a single disparate strong antigenic determinant, immunological tolerance demands the presence of all of the major foreign determinants.

Antigenic materials can elicit responses in immune individuals presensitized with tissue grafts. Intradermal challenge of sensitized guinea pigs with antigens derived from the donor strain yields direct, delayed-type skin reactions. Furthermore, sensitized peripheral lymphocytes preincubated

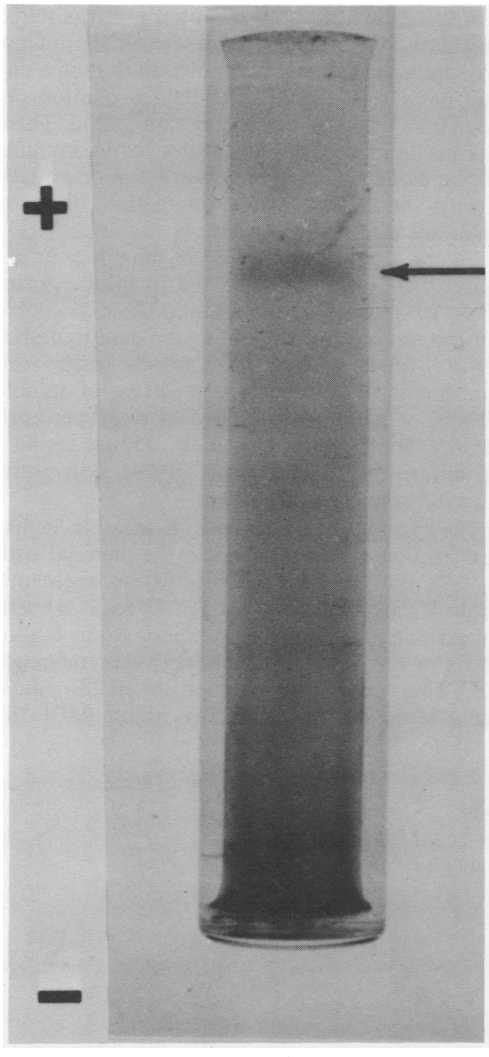

FIG. 5. *Reelectrophoresis of the active component. The antigen was purified by acrylamide gel electrophoresis (Fig. 4A) and then recentrifuged in another 7.5% gel in the presence of 8 M urea. A single well-defined component was revealed by Coomassie Blue staining. There was no evidence of heterogeneity (57).*

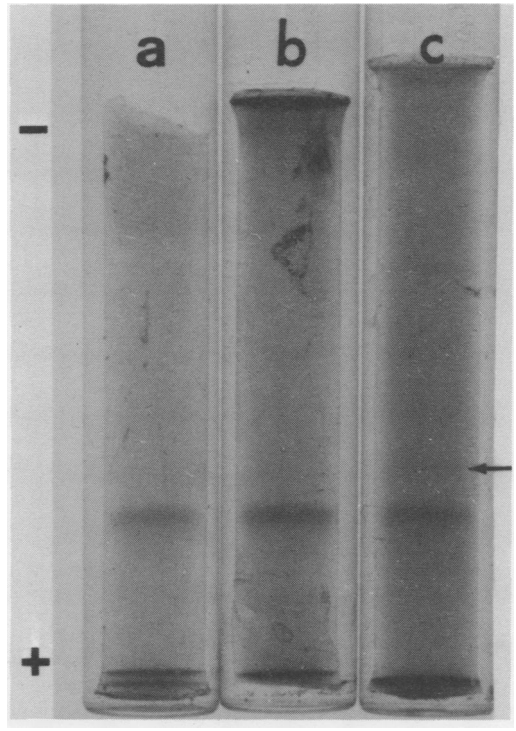

FIG. 6. *Transplantation antigens of different organs Biologically active materials purified from (a) spleen, (b) lung, and (c) kidney were recentrifuged on 7.5% acrylamide gels in the presence of 8 M urea. The same electrophoretic component was found in all three organs. A minor contaminant (arrow) was observed in the kidney extract (57).*

with donor antigen display transfer reactions when inoculated intradermally into third-party hosts or into irradiated hamsters, and they undergo blast transformation in vitro. Humans presensitized against histocompatibility substances by blood transfusions or by leukocyte injections display Arthus reactivity when confronted with intradermal antigen. Thus, preparations of transplantation antigens evoke immune expressions by lymphocytes derived from hosts presensitized solely with allogeneic grafts. These specific responses suggest that during the early phases of recognition of a foreign graft the host develops immunity to the very antigenic determinants which are present in the soluble extract.

Foreign tissue grafts also induce the production of humoral alloantibody with hemagglutinating, leukoagglutinating, hemolytic, and cytotoxic properties (100). Although humoral antibody plays an uncertain role in allograft rejection, extracts with transplantation antigenic activity readily stimulate the synthesis and inhibit the in vitro reactions of specific alloantibody.

Three biological activities—graft compatibility, delayed-type hypersensitivity, and humoral alloantibody—form the basis for the detection of antigenic determinants related to transplantation individuality.

Assay Systems Based upon Graft Compatibility

The second-set phenomenon is the foundation of the immunological hypothesis of graft rejection. A few days after putative antigens are administered to allogeneic hosts (the incubation period), skin grafts from members of the antigen donor's strain and from a third-party strain are

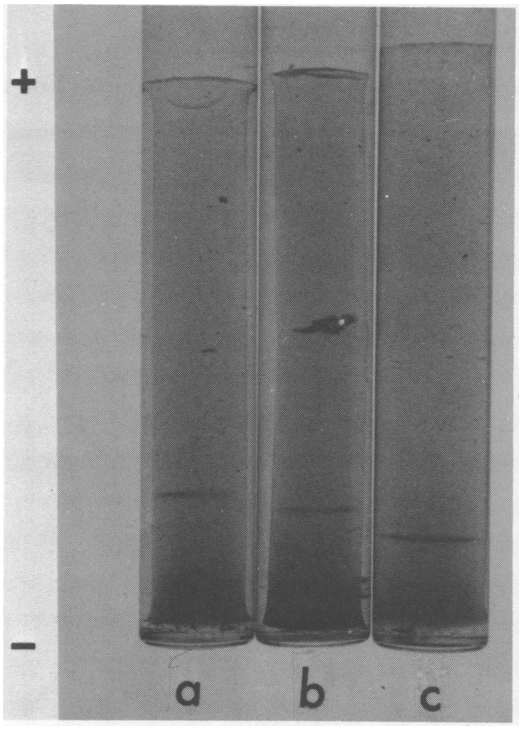

FIG. 7. *Effect of different gel concentrations, producing variable sieving conditions. Gel a has 15%, gel b 20%, and gel c 30% acrylamide in the sieving lower gel. There was no electrophoretic heterogeneity of the biologically active purified component.* [*Urea (8 M) was added to the lower gels to promote the dissociation of contaminants (57).*]

applied. The degree of test-graft epithelial destruction is estimated on the sixth day postgrafting. Grafts on nonimmune hosts show no epithelial destruction (first-set reactions), whereas grafts residing on hosts with preexistent immunity display stigmata of rejection (second-set reactions; Fig. 10).

The important variables in the immune responses obtained after antigen administration (100) include (i) the route of administration, with the subcutaneous route being more effacacious than other routes when inducing immunity and the intravenous route favoring tolerance; (ii) the dose of antigenic material (in logarithm cell equivalents), being linearly related to the percentage of epithelial destruction; and (iii) the length of the incubation period between antigen administration and challenge skin grafting, determining the potency of a given antigen dose. Figure 11 expresses the three-dimensional relationship of the factors: the logarithm of the number of cell-equivalents (antigen dose), the incubation period, and the degree of test-graft epithelial destruction. A fourfold greater dose of antigen was required to obtain accelerated rejection when the animal was challenged with a skin graft on the same day that he was sensitized (zero-day incubation period) than after a 4-day incubation period. There was less difference between the destructive immune responses engendered by large doses of antigen after 4- and 7-day incubation periods than by small doses of material.

Figure 11 reveals that 22% of the antigenic activity of the cells was obtained in soluble form; 48×10^6 cell equivalents of $105,000 \times g$ soluble antigen were required to obtain the same immunogenic effect as 10.7×10^6 sonically treated cell equivalents. Administration of 40 μg of soluble $105,000 \times g$ murine antigen yielded just perceptible immune responses, whereas 250 μg resulted in uniform 50% destruction of test skin grafts (Fig. 10; references 53, 131).

The immunogenic activity of guinea pig Sephadex fraction 1 was confined to the material with R_F 0.73 to 0.74. Electrophoretically homogeneous strain-2 transplantation antigen (1 to 3 μg) sensitized strain-13 animals to reject strain-2 allografts in accelerated fashion (Fig. 10; reference 60). This dose corresponded to 0.28 pmole per g of body weight, which is about 0.001 the

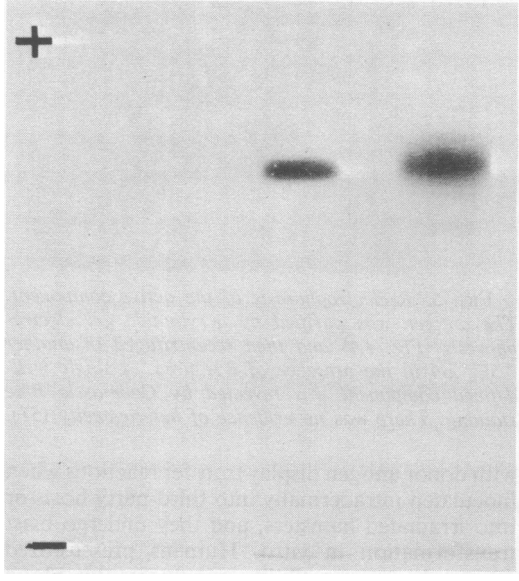

FIG. 8. *Reelectrophoresis of HL-A antigens. Histocompatibility antigen extracted from cultured human peripheral lymphocytes by sonic exposure was precipitated with ammonium sulfate and then was purified by preparative acrylamide gel electrophoresis. Reelectrophoresis in the presence of 8 M urea revealed a single band at concentrations of 50 μg (on the right) and 350 μg (on the left).* (*Reference 101.*)

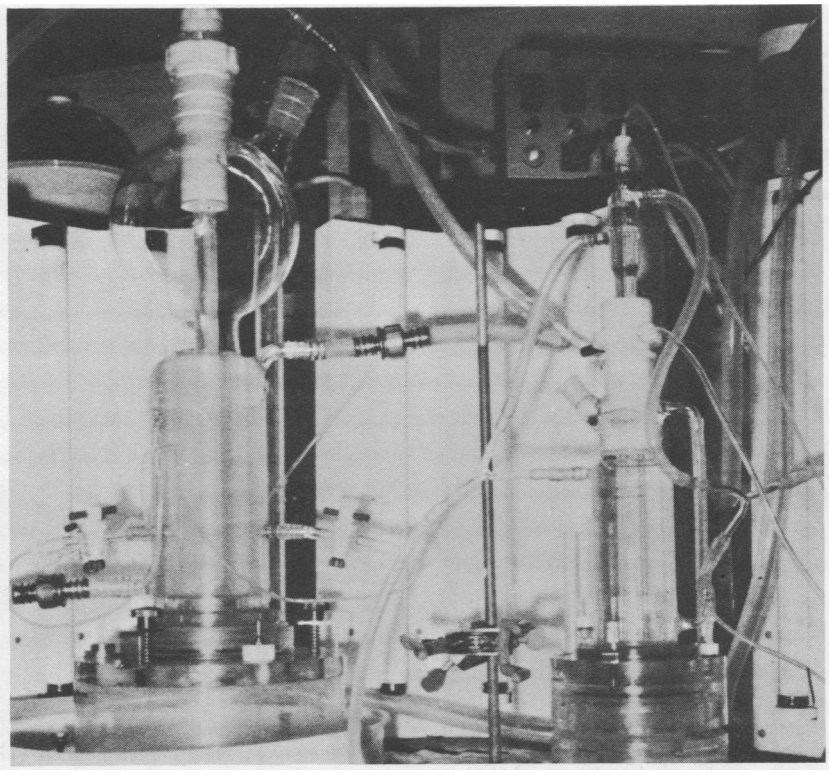

FIG. 9. *Equipment for preparative acrylamide gel electrophoresis. The initial run, using selective stacking conditions, separated a fraction containing the active component from the other 90% of the crude material (apparatus on the left). The final purification run was performed on the column at the right. Both pieces of equipment are available from Buchler Instruments, Fort Lee, N.J.*

minimum circulating antigen concentration to induce tolerance in most other immunological systems. After storage of the purified guinea pig antigen, 91% of the material remained in the monomer state (n = 1, molecular weight 15,000). Ultracentrifugal analyses showed that the other 9% was an aggregated moiety with molecular weight of 120,000 (n = 8). After prolonged storage, there was variable aggregation with n = 2, 4, 5, 8, 10, 15 (molecular weights up to 244,000; reference 61). Presumably, the small fraction of aggregated material was the immunogenic component, and a tolerant rather than an immune state would result from administration of pure monomer.

Assay systems based upon the second-set phenomenon have several disadvantages. First, they are tedious and time consuming, and they require relatively large amounts of antigen. Second, precise, semiquantitative epithelial scoring demands histological assessment, which is an art difficult to perform on a routine basis. Third, accelerated graft rejection requires that the antigen possess not only the antigenic determinant but also the biological properties necessary to convey immunogenicity. Solubilized antigens are generally poor immunogens compared with intact cells due to the special biological properties of cells: (i) their capacity to divide, increasing the actual dose; (ii) their homing tendency for lymph nodes; (iii) their resistance to catabolism; and (iv) their apparent enhanced immunogenicity related to the membranous location of the antigen or to the contribution of other cellular components as adjuvants. The immunogenicity of soluble antigens was not increased by admixture with cell membrane fractions or by emulsification in complete Freund's adjuvant. Therefore, although the second-set assay defines an extract as a transplantation antigen, it has limited application to the routine assay and standardization of tissue extracts.

Pretreatment with appropriate doses of cell extracts prepared by the sonic-treatment method prolongs the survival of donor-type allografts. Medawar's report (87) of extended survival of allogeneic skin grafts after administration of semisoluble materials has been confirmed with soluble,

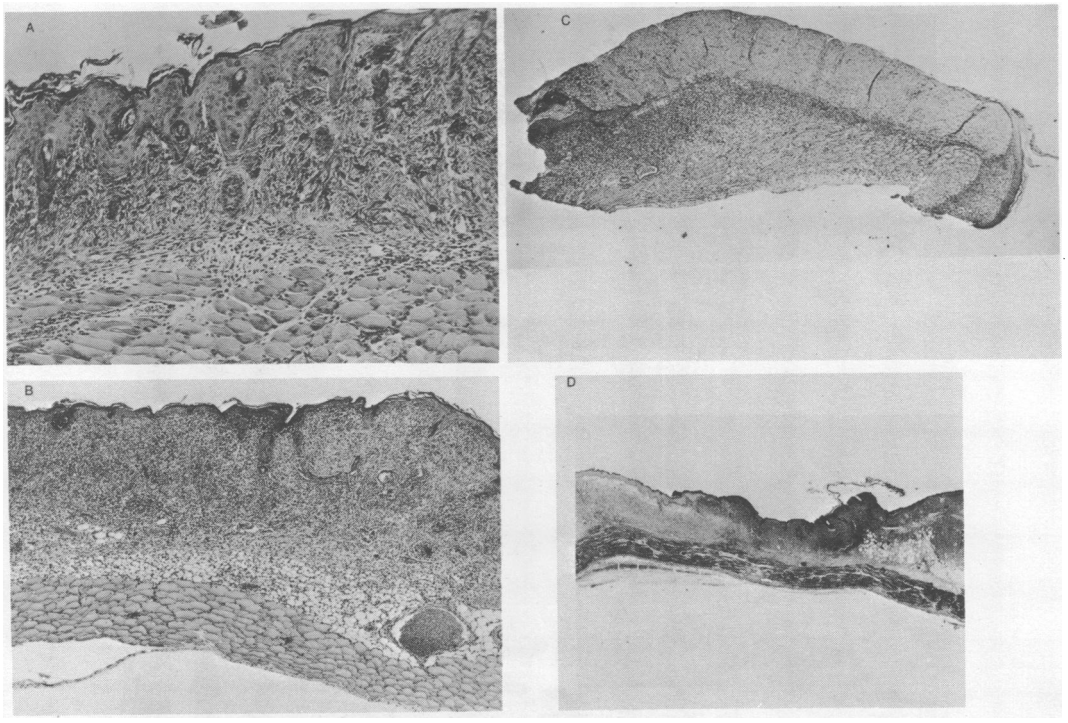

FIG. 10. *Histological appearances of four grafts 6 days after transplantation. (A) First-set murine graft on a normal nonsensitized allogeneic host; there is hyperplastic epithelium and a clear dermal field. (B) Graft was applied onto an allogeneic host that had received 50 μg of Sephadex fraction I donor transplantation antigen. There is epithelial destruction, an abundant mononuclear infiltration in the dermal field and in the perivenous areas, as well as vascular congestion and hemorrhage. (C) Graft applied to an allogeneic murine host that received 250 μg of Sephadex fraction I-soluble murine antigen. The graft failed to establish primary connections with the host—"the white graft reaction." (D) Strain 13 guinea pig was treated with 3 μg of acrylamide-purified strain 2 antigen 4 days prior to the application of this strain 2 graft, which shows a hyalinized appearance with no evident epithelial survival.*

sonically treated transplantation antigens by Wilson and colleagues (129, 130). When 1 to 7.5 mg of ultracentrifugal supernatant fluid per kg was given as a single dose, challenge-donor renal allografts were rejected in accelerated fashion. If the hosts were treated with divided antigen doses for 14 days prior to transplantation and with azothioprine and methylprednisone or antilymphocyte serum after operation, the allografts had prolonged survival. Some kidneys were accepted for 1 year, and frequently the host did not require immunosuppression during 100 days of this period. The effect was uniform; all hosts that received antigen had prolonged survival. On the other hand, hyperimmunization with high doses of antigen divided over periods of 4 to 6 weeks yielded immune responses, including lymphocyte-transfer reactions and circulating alloantibody.

Similar results were obtained in rats (129). Kidneys allografted to Lewis rats which had been pretreated with 3 to 4 mg of sonically treated, solubilized (L × BN) F_1 liver cell antigen per kg for 20 to 40 days preoperatively survived up to 60 days without immunosuppressive agents. In some instances, a single injection of 4 mg/kg yielded 85-day survivals. Zimmerman (129) found that a single dose of 60×10^6 cell equivalents of sonically treated ultracentrifugal supernatant antigen administered on the day of transplantation uniformly prolonged renal allograft survival to 40 days. Divided doses given over a 1-week period preoperatively prolonged survival to 100 days. It is not yet clear whether the prolonged survival was due to enhancement by blocking alloantibody, to true immunologic unresponsiveness, or to immune deviation.

A range of biological responses to graft challenge can be induced by pretreatment with antigens solubilized by sonic treatment. The prospective host displays immunity or prolonged survival of donor grafts, depending upon the total dose, the treatment schedule, and the route of antigen administration.

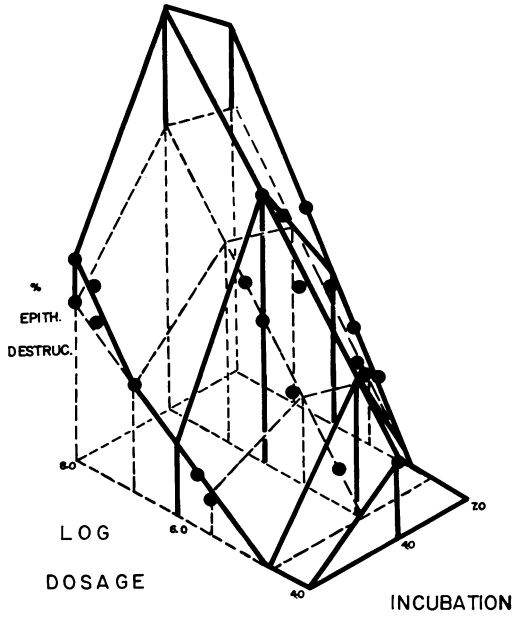

FIG. 11. *Three-dimensional figure quantitatively expressed the relationship among the dose of antigen (in logarithm cell equivalents), the interval between antigen injection and skin graft challenge (the incubation period), and the degree of challenge graft epithelial destruction in the mouse. Closed lines represent the sensitization pattern obtained with the membranous antigen of intact spleen cells. Interrupted lines show the result with sonically treated solubilized antigen. A 4-day incubation period between antigen administration and skin graft challenge yielded the greatest degree of epithelial destruction; however, this effect was less pronounced at high antigen dosages.*

Systems Based upon DTH Responses

A considerable body of evidence suggests that allograft immunity is mediated by DTH. The DTH methods used to study a variety of antigens are applicable to the assay of soluble transplantation determinants. These techniques are quite sensitive because the responses which they detect reflect the amplification by a large number of indifferent cells of a basic recognition phenomenon occurring between small numbers of immune cells and the sensitizing antigen.

Cell mediated immune responses. Three in vivo cell-mediated DTH reactions have been used to demonstrate allograft immunity: direct reaction, transfer reaction, and irradiated hamster test. Cutaneous DTH reactions are characterized by the slow progression of the induration, the mononuclear infiltrate, the lack of correlation between the reaction and antibody responses, and the capability of cells, but not serum, to transfer passively reactivity (109). Brent et al. (13) reported that animals presensitized with skin allografts developed cutaneous reactions when challenged with allogeneic donor cells: the direct reaction. They found that 3 mg (10^6 cell equivalents) of a crude fraction prepared by brief ultrasonic destruction of water-lysed spleen cells gave a perceptible response and that 12.5 mg (10×10^6 cell equivalents) gave a definite DTH response.

Direct reactions were used to identify the active fractions during the purification of guinea pig transplantation antigens solubilized by sonic treatment (55). Normal (nonimmune) and presensitized allogeneic hosts were challenged with Sephadex G-200 fraction 1. Whereas normal animals never developed cutaneous reactions to 100 μg of material, sensitized hosts had graded responses to 10 to 250 μg of donor-type antigen. Of the 72 animals challenged with 10 μg or more of soluble antigen, 87% showed a positive response. The cutaneous reactions had the temporal and histological (Fig. 12) characteristics of DTH responses. Furthermore, this reactivity was passively acquired by adoptive transfer of lymph node cells derived from hosts presensitized with donor-type allogeneic skin grafts.

The direct reaction was particularly useful in detecting the active principle among the 20 components resolved by PAGE (57). The R_F 0.73 to 0.74 component elicited DTH responses at doses of 0.1 μg and was later shown to immunize allogeneic hosts against donor-type skin grafts.

The DTH responses were observed in humans who had been immunized by intradermal leukocyte injections 7 to 12 days prior to challenge with soluble antigen. Similar reactivity had been seen by challenge of sensitized hosts with intact lymphocytes (84, 88).

Immediate cutaneous reactions were elicited in humans who had been sensitized with blood transfusions or multiple leukocyte injections (or both) and who were actively producing cytotoxic alloantibody. When these individuals were challenged with soluble antigen possessing the corresponding HL-A determinants, they developed Arthus-type cutaneous responses, characterized as violaceous reactions appearing within 6 hr as flat hemorrhagic sites. Similar reactions have been elicited in rabbits sensitized with dissociated epidermal cells in Freund's adjuvant and challenged with leukocytes, and in guinea pigs sensitized with splenic cells emulsified in Freund's adjuvant and later challenged with intact cells or with subcellular sonically treated antigen. Local passive Arthus reactions have been obtained by the inoculation of soluble antigen into intradermal sites on guinea pigs that had received passive transfer of alloantibody raised by hyperimmunization with spleen cells in Freund's adjuvant.

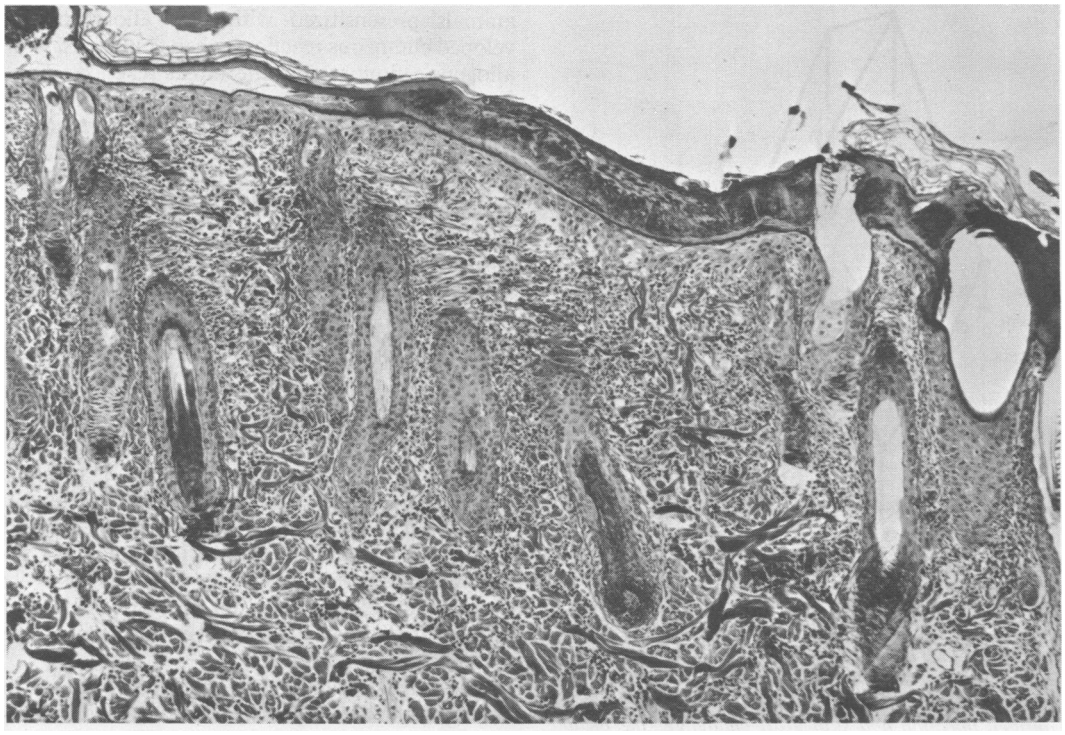

FIG. 12. *Histology of the direct reaction. The histological section shows a reaction site in which 30 μg of strain 2 transplantation antigen was deposited intradermally into a strain 13 host that had rejected a donor-type skin graft 10 days prior to challenge (× 65). Abundant mononuclear infiltrate, disruption of collagenous bundles, separation of overlying epithelium with central necrosis, and perivenous accumulations (56) occurred.*

Clark and Munoz (20) extended the observations of Arquilla and Finn (6) on the immunological responses of guinea pigs to insulin. They applied direct cutaneous reactions to elucidate the molecular distribution and conformational interactions of insulin antigenic determinants. Humoral antibody formation was stimulated only by the intact insulin molecule and not by isolated chains, suggesting that antibody synthesis required the conformation of A and B chains to define the antigenic determinants. Similarly, the isolated peptide chains did not elicit passive cutaneous anaphylaxis at doses 200-fold greater than those required for the intact molecule. On the other hand, DTH was elicited by either the intact molecule or the B chain, but not by the A chain. Selective desensitization experiments with the intact molecule and the B chain revealed that each moiety possessed specific antigenic determinants which could not be desensitized by cross-reaction. In analogous fashion, fragments of transplantation antigens might be used in direct reactions to clarify the determinants of allograft immunity.

Brent et al. (13) demonstrated that the intradermal inoculation of immune peripheral lymphocytes into a member of the sensitizing strain evoked a cutaneous DTH response: the transfer reaction. The reaction was presumably due to the interaction between sensitized cells and allogeneic antigen residing within or brought to the skin-test site. The third-party test was devised for the assay of transplantation antigen. In general outline, strain A lymphocytes derived from a host previously sensitized with an allogeneic strain B donor skin graft were preincubated with subcellular strain B antigen, and the mixture was then inoculated into the skin of a nonimmune strain-A host. This maneuver planted sensitized cells and homologous donor antigen in an isogeneic, but nonimmune, environment. Combinations of 10×10^6 sensitized cells and 50 to 100 μg of Sephadex fraction 1 allogeneic antigen displayed vigorous DTH reactions, containing prominent mononuclear and polymorphonuclear exudates forming large indurated areas and resulting in necrosis of overlying isogeneic host-skin elements and in persistent alopecia. These reactions occurred only when immune cells were admixed with corresponding donor antigen, and not with combina-

tions of sensitized cells and isogeneic antigen or with normal lymphocytes and allogeneic antigen (55).

The irradiated hamster test permits immunocompetent cells to interact in vivo with soluble antigen in an immunologically incompetent host, as opposed to third-party tests wherein the host, although nonimmune, does contribute to the cellular infiltrate responding to the mediators of DTH. After irradiation with 1,500 r, hamsters were inoculated with admixtures of 75 μg of Sephadex fraction 1 guinea pig transplantation antigen and 20×10^6 sensitized lymph node cells. The resulting cutaneous nodules in the thin dorsal skin were scored on the basis of hardness. Antigen with allogeneic cells displayed immune reactions, whereas nodules failed to develop at sites containing antigen and syngeneic cells (63). Equal degrees of hardness were observed when the transplantation antigens were provided as 10×10^6 intact cells or as 75 μg of soluble material. In both cases, the nodules reached a maximum hardness within 24 hr and faded rapidly after 48 hr. Reaction sites containing two populations of nonimmune, allogeneic guinea pig lymph node cells showed indolent development and persisted for the 4-day observation period (63).

Cell-mediated immune responses. Transplantation antigens can be detected by stimulation of in vitro blast transformation. Lymphocyte transformation, which consists of increased phospholipid turnover, phagocytosis, ribonucleic acid and deoxyribonucleic acid (DNA) synthesis, and cell division, can be monitored by microscopic examination of stained cell smears, by radioautography, or by pulse labeling with ^{3}H-thymidine to estimate the rate of DNA synthesis. Factors which trigger lymphocyte transformation include (i) nonspecific mitogens [e.g., phytohemagglutinin (PHA) and pokeweed]; (ii) antibodies directed against immunoglobulins or against components of the cell surface; (iii) allogeneic cells, which may elicit a primary immune response or may reawaken a latent reactivity; and (iv) specific antigens to which the host has been presensitized.

Blast transformation is a manifestation of DTH (89, 132). Zweimann and Phillips (132) showed that guinea pigs sensitized to tuberculin concomitantly developed in vivo skin reactivity and in vitro lymphocyte transformation. Pretreatment with 6-mercaptopurine interfered only with the expression of the cutaneous response, either by suppressing the generation of reactive cells or by altering the in vivo conditions, e.g., by depleting the short-lived bone marrow mononuclear cells which nonspecifically migrate to the DTH site (72, 122, 123).

Although in vitro reactions do not depend on these nonspecific cells, they do require macrophages in addition to responsive lymphocytes. Mixed cultures of allogeneic pure lymphocyte suspensions show considerably less mitotic activity than crude leukocyte suspensions (51, 94). The diminished reactivity of pure lymphocyte suspensions, prepared with cotton-packed syringes, nylon stockings, or glass bead columns, was restored by the addition of macrophages which were purified by subculture of leukocytes exposed to 2,000 r or by albumin flotation (110). The role of the macrophage was nonspecific; no experience with the antigen was needed to reconstitute the reactivity of the lymphocyte cultures. Schechter and McFarland (110) found that macrophages pulsed with purified tuberculo-protein derivative (PPD) could transfer the antigenic message to sensitized lymphocytes not exposed to tuberculin. After a 4-day period between exposure to tuberculin and addition to lymphocyte cultures, the macrophages lost the message, presumably due to metabolism. The macrophages might "process" the mitogen, might concentrate the antigen on their surface, increasing its availability to the lymphocyte, or might mobilize a cell-bound lymphocyte substance, possibly through intercytoplasmic bridges.

Sensitized guinea pig peripheral leukocytes expressed allograft immunity in vitro after stimulation with allogeneic Sephadex fraction 1. Strain 13 cells, derived from hosts preimmunized with strain-2 skin allografts, incorporated 6.7-fold more thymidine on the 3rd day after exposure to 75 μg of allogeneic strain-2 antigen compared with the same amount of syngeneic strain-13 antigen (63). Cultures pulsed on the 6th day showed significantly less incorporation. The diminished incorporation did not appear to represent a toxic effect but rather a nonspecific depression of mitosis by the extracts, similar to the cases observed by Lowenstein (76) and by Jones et al. (52). The latter group found that an extract of fresh pig lymph nodes inhibited DNA synthesis in PHA-stimulated normal human peripheral blood leukocytes. Since no direct cytotoxic effect was apparent, they proposed that the depression was caused by a direct action on the cells or by competition with some factor(s) in the culture medium.

Primary induction of immunity in vitro. The mixed lymphocyte culture (MLC) reaction, the mitotic response of nonsensitized, allogeneic lymphocyte populations admixed in vitro, is related to the histoincompatibility of the donors. A statistical method has been designed to classify the degree of incompatibility between donors based upon their interactions in mixed cultures (3). The MLC phenomenon might represent an induction of allograft immunity or a recrudesence of a latent hypersensitive state engendered from

previous exposure to cross-reacting neoplastic or infectious agents (28, 37, 83, 116).

Lymphocytes exposed to allogeneic cells in vitro not only undergo blast transformation but also acquire specific cytotoxic immunity, demonstrated by their agglutination to (69) and by their release of radiochromium label from (44) donor-type target cells. Manson and Simmons (82) found a "primary" in vitro response to a subcellular fraction as a source of allogeneic transplantation antigen. Lymph node cells incubated with allogeneic microsomal lipoprotein preparation (MLP) had a twofold enhanced incorporation of radiolabel. Although a similar level of stimulation had previously been observed when normal guinea pig peripheral lymphocytes were exposed to allogeneic, soluble, sonically treated transplantation antigen, this result was just outside the 95% confidence limits delineated with 25 replicate cultures exposed to syngeneic antigen. However, Manson and Simmons further demonstrated that lymph node cells incubated with DBA-2 MLP had acquired specific immune capabilities: they could kill DBA-2 plaque-forming cells in the Jerne assay. Thus, the inductive "primary" sensitization phase and the expressive destructive phase of the allograft reaction can now be studied in vitro with subcellular transplantation antigens.

Pharmacological mediators of hypersensitivity. Immunologically activated cells undergoing DTH responses release a set of pharmacologically active substances: (i) migration inhibition factor (MIF), (ii) mitogenic factor (MF), (iii) leukotactic factor, (iv) lymphotoxin (LT), (v) a factor causing DTH reactions in normal animals (11), (vi) an interferon-type activity, and (vii) a heat-stable dialyzable polynucleotide with adjuvant activity (92). Although the production of these effector molecules results from exposure of sensitized lymphocytes to specific antigen, the effectors act nonspecifically on all target cells and do not require the persistence of the antigen. Sensitive methods are now available to detect these substances which function to amplify the response of immunocompetent cells by affecting nonspecific leukocytes and target cells. The direct measurement of the generation of effector molecules might become a valuable tool for transplantation antigen-assay techniques.

In 1932 Rich and Lewis (105) described the inhibition of migration of lymphoid cells when cultured in the presence of the sensitizing mycobacterial antigen. Migration inhibition is an in vitro correlate of DTH, since lymphocytes from individuals possessing immediate hypersensitivity do not show this response. Migration inhibition has been demonstrated in a number of immune situations, e.g., with infectious agents (27, 31, 106, 111, 120) and in autoimmune diseases (106). The interaction of sensitized cells and antigens appears to release MIF, which produces an effect upon the macrophage (25). The MIF might serve to immobilize mononuclear cells at the site of antigen deposition, contributing to the formation of the nonspecific infiltrate seen in DTH cutaneous reactions.

The capillary tube method of George and Vaughn (36) provided a significant advance in MIF system technology. After exposure to antigen, sensitized guinea pig lymphocytes inhibit the migration of nonsensitized peritoneal exudate macrophages from the capillary tube. In the initial work of Al-Askari et al. (1, 2), donor-type allogeneic cells inhibited the migration of peritoneal exudate cells derived from presensitized animals. Exposure of presensitized lymphocytes to donor-type particulate transplantation antigen prevented the migration of indifferent peritoneal exudate cells. The migration of macrophages in mixtures of peritoneal exudates derived from two allogeneic mouse or guinea pig strains was not affected unless one of the strains had been presensitized by a skin graft from the other. Similarly, random mixtures of allogeneic human leukocytes showed migration inhibition only in a single patient who had received multiple transfusions and presumably was sensitized against HL-A antigens.

LT is released by sensitized cells in contact with specific antigen and results in target-cell destruction (42, 45, 107). Ruddle and Waksman (108) added tuberculin to sensitized rat lymph node cells and inhibited the growth of allogeneic or syngeneic fibroblasts in the same culture. Heise and Weiser (45) showed that only sensitized lymphocytes produced cytotoxins. The cytotoxins acted nonspecifically since syngeneic, allogeneic, and xenogeneic cells were susceptible to LT, which was thought to destroy allografts in transplant-rejection phenomena.

Ward et al. (127) found a leukotactic factor in the supernatant fluids of sensitized lymphocytes exposed to homologous antigen in vitro. Culture supernatant fluids were added to the bottom chamber of a system containing rabbit mononuclear cells in the upper chamber. Enumeration of the number of cells migrating toward the lower chamber measured the leukotactic activity of the supernatant fluid. The authors proposed that leukotactic factor allowed a few sensitized lymphocytes to elicit the accumulation of a large number of nonsensitized mononuclear cells at the DTH site.

Immune leukocytes exposed to the sensitizing antigen release MF, which stimulates the proliferation of normal leukocytes in vitro. Spitler and Fudenberg (114) demonstrated MF in super-

natant fluids derived from cultures of sensitized cells incubated with PPD. Although PPD itself failed to stimulate nonsensitized cells, the supernatant fluids had a mitogenic effect on normal allogeneic or syngeneic cells and thus did not represent transplantation antigens. The authors suggested that MF explained how a small number of activated cells recruited a proliferative response from a large number of nonsensitive cells.

Valentine and Lawrence (125) observed that supernatant fluids derived from cultures of sensitized cells activated by 36-hr contact with tuberculin increased the thymidine incorporation by normal leukocytes 4- to 25-fold. Leukocyte proliferation was not affected by PPD without supernatant fluid or by supernatant fluids obtained from cultures of sensitized cells not exposed to PPD. Increasing the amount of PPD in the active supernatant fluid yielded a greater mitotic response, suggesting that the transferred material was not a simple mitogen but that it contained immunological information. The specificity of this effect distinguished it from the nonspecific mediators of DTH. The relationship between the transfer factor with the mitogenic effect in vitro and the transfer factor liberated from blood leukocytes and carrying immunologic information in vivo is unclear.

The pharmacological activities mediating DTH might represent distinct chemical moieties or multiple actions of a single substance in different assay systems. Spitler and Fudenberg (114) found that guinea pig MF, MIF, and leukotactic factors were all heat stable. On the basis of heat lability and separation on Sephadex gel filtration and sucrose gradients, Ward et al. (127) dissociated leukotactic factor from MIF. Chemical characterization of these substances might not only clarify the mechanism of tissue destruction in DTH but also provide tools to measure an amplified product of the activation of sensitized cells by purified transplantation antigens.

In summation, DTH assays afford rapid, sensitive, and reproducible methods for the identification of transplantation antigens. Techniques demonstrating primary sensitization and immunodepression in vitro may yield information similar to that derived from cumbersome assays based upon the compatibility of allografts. The mediators of DTH may offer new tools to measure sensitively the response of immune cells to soluble transplantation antigens in vitro.

Systems Dependent upon Humoral Alloantibody

The two types of humoral antibody assays are the induction of alloantibody production directed against the determinants of the antigen donor and the in vitro inhibition of the action of alloantisera raised with donor-type target cells or tissues.

Murine alloantigens solubilized with sonic treatment induced the formation of hemagglutinins and inhibited the action of these antibodies in vitro (43). The application of hemagglutinating, leukoagglutinating, hemolytic, and cytotoxic antibody inhibition assays in animals has been reviewed (100).

Whereas in animals the other assay techniques can be readily applied to derive information about the effects of antigens in biological systems more directly reflecting allograft rejection, in humans inhibition of alloantibody has been the most convenient method to detect HL-A antigens. The tissue extracts with in vitro activity against HL-A alloantisera have not yet been proven to be true transplantation antigens, i.e., substances which specifically affect donor-type skin grafts. The apparent relevance of the HL-A specificities to histocompatibility justifies the full exploitation of serological techniques to aid in the purification of antigens suitable for clinical testing in man.

Human histocompatibility antigens can be standardized by their capacity to inhibit alloantibody in vitro. Although immune cytolysis can be detected by the release of radiolabel from appropriate target cells, the microdroplet technique of Terasaki and McClelland (118) is more widely applied by serologists. This system was modified to quantitate antigen inhibition (64). Antigen dissolved in 0.001 ml of 0.9% NaCl (*p*H 7.4) was incubated for 1 hr at 25 C with 0.001 ml of alloantibody at zero, two, or four cytotoxic units. Target cells (as 3,000 peripheral lymphocytes) were added to the microdroplets together with 0.003 ml of rabbit complement. After a 4-hr incubation period, the reactions were terminated with 0.001 ml of 36% Formalin. The viability of the target cell population was assessed by inverted phase microscopy. Dead cells displayed a prominent nucleus with clumped chromatin and were readily distinguished from viable cells.

Sonically treated soluble antigens prepared from human spleens (64) and fetal tissues (96) inhibited specific HL-A alloantisera in a pattern consistent with the direct and absorption phenotypes of the tissue donor. There was a 10% incidence of anomalous reactions which were of two kinds: (i) typing-absorption-positive-antigen-negative (TAPAN) reactions which have been attributed to a subliminal concentration of antigenic determinants in the preparation; and (ii) typing absorption-negative-antigen-positive (TANAP) reactions due to latent multispecificity of the antisera, to antigenic cross-reactivities within the HL-A system, or to tissue-specific factors. The HL-A 3 and HL-A 5 determinants showed the greatest incidence of anomalous reactions; indeed, these determinants are the most difficult to define

TABLE 2. *Correspondence of the cytotoxic inhibitory activity of alloantigens with the phenotype of the cell line donor*

HL-A specificity	Test serum	RPMI 1788		RPM 7249		RPMI 4098		WIL-2	
		Donor typing	Antigen inhibition	Donor typing	Antigen inhibition	Donor typing	Antigen inhibition	Donor typing	Antigen inhibition
1	32.19	−	−	+	+	−	−	+	
2	11.03	+	+	+	+	−	−	+	+
	Pinquette	+	+	+	+	−	−	+	
3	Storm	−	−	−	−	+	+	−	
5	D66-6222-V	−	−	−	−	−	−	+	+
7	Cutten	+	+	−	−	−	−	−	−
	Hutter	+	+	−	−	−	−	−	
8	Chayra	−	−	+	+	−	−	−	

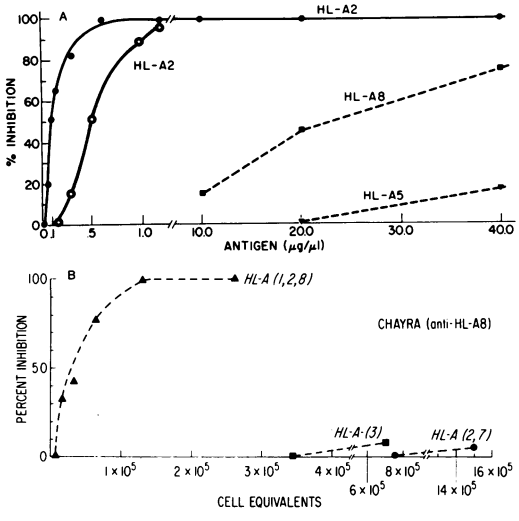

FIG. 13. *Arithmetic plots of antigen inhibition. (A) Arithmetic plot of degree of inhibition of immune cytolysis in microdroplets versus amount of RPMI 1788 (HL-A 2+, 5−, 8−) 30% ammonium sulfate precipitate antigen preincubated with two cytotoxic units of alloantibody. There was inhibition of anti-HL-A 2 TO 11.03 (●) and Pinquette (○), but not of anti-HL-A 5 D-66 (▼) or of anti-HL-A 8 Chayra (■). (B) Alloantiserum Chayra (anti-HL-A 8) was preincubated with serial amounts (in cell equivalents) of antigen from RPMI 7249 (HL-A 8+, ▲), from RPMI 4098 (HL-A 8−, ■), or from RPMI 1788 (HL-A 8−, ●). Inhibition was only obtained with RPMI 7249 (101).*

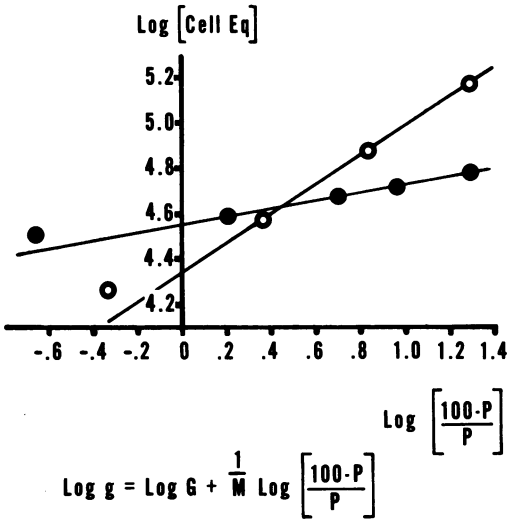

FIG. 14. *Effect of the alloantibody on the van Krogh plot. Two alloantisera recognizing HL-A 2 (TO11.03, ○ and Pinquette ●) were preincubated with serial amounts of RPMI 1788 ammonium sulfate precipitate (expressed as log cell equivalents) and then were tested for cytotoxic activity against peripheral lymphocytes of donor A. Alloantiserum TO11.03 had a significantly greater sensitivity in the inhibition assay; its ID_{50} is lower than that of Pinquette and its avidity coefficient is several-fold greater than that of the other alloantiserum.*

accurately with monospecific serological reagents.

On the other hand, the antigens prepared from cultured lymphocytic lines RPMI 1788, RPMI 4098, RPMI 7249, and WIL-2 inhibited specific alloantisera in a pattern exactly corresponding to the phenotype of the cell-line donor (103). Table 2 reveals that the antigenic materials possessed mosaics of specificities HL-A 1, 2, 3, 5, 7, 8.

Plots of percentage cytotoxic inhibition versus dose of antigen on arithmetic scale were sig-

moidal (Fig. 13). Since the antigen inhibition was inversely proportional to the amount of antibody, the data were transformed according to the Reif modification of the van Krogh equation (99): log g = log G + 1/M [log (100 − P)/P], where g is the ratio of the total quantity of antigen to that of antiserum, P is the potency of the antiserum remaining free in the supernatant fluid after absorption, G equals g when P is 50%, and 1/M is the slope. A low value of 1/M indicates sharp absorption. A quantitative description of several important parameters was then possible.

The y-intercept of the van Krogh plots (log G) represented the 50% inhibition dose (ID_{50}), i.e., the dose of material required to reduce the cytotoxic effect of the antiserum to half that of sera not preincubated with antigen. The ID_{50} value depended not only upon the potency of the alloantigen, but also upon the alloantibody being used as the cytotoxic reagent (Fig. 14, 15). In Fig. 15, employing the target cells of donor B, the ID_{50} value of sonically treated HL-A antigen with alloantiserum Pinquette is 7.5-fold greater than the ID_{50} value of the same antigen with alloantiserum TO11.03; (both sera recognize HL-A 2). Several factors may affect the performance of alloantibodies. (i) Latent polyspecificity of the serum would increase the ID_{50} value since the antigen would not interfere with the action of contaminant antibodies directed against other specificities. (ii) Susceptibility of the serum to nonspecific factors present in the antigen extract would decrease the ID_{50} value. (iii) Differences in the relative avidity of alloantibody for antigenic determinants arrayed on soluble materials as opposed to the same (or at least similar) determinants displayed on intact surfaces would also affect performance. The serum used in the assay must be denoted when stating the ID_{50} value of an antigenic preparation.

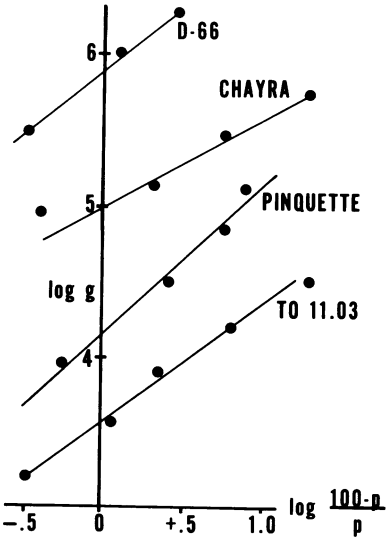

FIG. 15. *Specificity ratio. The inhibitory activity of RPMI 1788 ammonium sulfate antigens against TO 11.03 and Pinquette (both homologous anti-HL-A 2 antisera) and Chayra and D-66 (both indifferent antisera) was plotted on van Krogh coordinates. The maximal difference in ID_{50} units was 2.4 log units (i.e., 250-fold) between D-66 and TO11.03. There was no significant difference in the results obtained with five alloantigenic preparations tested against these antisera.*

Table 3 summarizes the optimal yields of soluble material inhibiting the cytotoxic action of TO11.03. From the cell line RPMI 1788, the 3 M KCl method yielded more antigen than did sonic treatment. Although the activity of KCl-solubilized material in compatibility and DTH assays has not been established, this method does afford greater yields of serological inhibitor from human lymphoid cell lines. The active principle from the sonic treatment and KCl methods appears to be chemically similar.

The ID_{50} value provides an index of purification if the same antiserum is used to measure antigenic activity. With alloantiserum TO11.03, there was a 25-fold ID_{50} purification from the crude sonically treated state to the homogeneous polyacrylamide component (Table 4).

The van Krogh factor (M), the avidity coefficient (AC) of the antigen-antibody complex, reflects the affinity of the antigen for antibody. AC is an extremely sensitive parameter of solubilized antigen activity. It increases 6,400-fold in the course of purification of the active principle (Table 4).

Although the ID_{50} and AC are excellent indexes of the inhibitory activity of sonically treated antigens, the *immunogenic* potency is more directly related to the specificity ratio (SR) of the materials. The SR was defined as the concentration of antigen required to inhibit an antiserum directed against an antigenic determinant not

TABLE 3. *Optimal solubilization yields*

Methods	Amt of protein/ 10^9 cells (mg)	ID_{50} (units/ mg)	Maximal total ID_{50} units/ 10^9 cells
Sonic treatment.....	40	10,000	400,000
Detergent-SLS.....	24	7,000	168,000
3 M KCl.....	35	15,000	525,000
Sonic treatment-KCl[a]	4	33,000	132,000

[a] Pellet remaining after ultracentrifugation of sonic-treated material was reextracted with 3 M KCl for 16 hr, and the extract was subjected to ultracentrifugation.

TABLE 4. *Purification of human histocompatibility antigen*

Purification stage	Amt of protein/10^8 cells (mg)	ID_{50} Units/mg	Total ID_{50} units	ID_{50} Purification	Specificity ratio	Avidity units	Avidity purification
Ultracentrifugal supernatant fluid	40.0	4,000	160,000	1.0	25–33	70	1
$(NH_4)_2SO_4$ ppt (30%)	12.0	10,000	120,000	2.5	240–300	3,500	50
Acrylamide electrophoresis	1.0	100,000	100,000	25.0	—[a]	450,000	6,400

[a] Impossible to concentrate enough material to determine the level of nonspecific inhibition with the level of the acrylamide fraction.

present in the antigen donor ID_{50}^{I} versus the concentration required to inhibit an antiserum directed against a determinant which is present in the donor (ID_{50}^{H}); SR = ID_{50}^{I}/ID_{50}^{H}. The ratio expresses the specific inhibitory power of an allogenic preparation against the homologous as opposed to an indifferent antiserum.

The SR depends upon three important factors. (i) Although the ID_{50} required to inhibit the homologous antibody is proportional to the amount of antibody employed in the test, nonspecific inhibition of the indifferent serum by large antigen doses was rather independent of the antibody dose. Thus a higher SR was obtained by using homologous antibody at titer level (zero cytotoxic units) than at two or four cytotoxic units. (ii) The choice of the antiserum was crucial since the ID_{50} value of an antigenic preparation depended upon the serum. Choice of a relatively sensitive homologous serum and a relatively insensitive indifferent serum resulted in an SR one order of magnitude greater than with an insensitive homologous serum and a sensitive indifferent reagent. Figure 15 illustrates van Krogh plots for several alloantisera that were preincubated with RPMI 1788 (HL-A 2+,5−,8−) soluble, sonically treated antigen. There was a 2.4 log unit difference between the ID_{50} values of D-66 (anti-HL-A 5), an indifferent serum, and TO11.03, the homologous serum, yielding an SR of about 250. The target cell was the third critical factor. The ID_{50} value depends upon the sensitivity of the target cell to immune cytolysis (*compare* Fig. 14 and 15). The same donor's cells must be used for the cytotoxic reactions of the homologous and indifferent antisera. Although the antigen donor's peripheral lymphocytes are most sensitive to the blocking action of his own antigen, they cannot define the SR of the antigen since there is no antigenic determinant present on his cells and not in the extract (103). The target cell effect might stem from differences in the susceptibility of cells to complement-mediated lysis, to latent multispecificities in the alloantibodies, to variable expression of the antigenic determinants on different cell surfaces, or to epistatic interactions with other histocompatibility or unrelated cell surface specificities.

TABLE 5. *Yield of soluble antigen by KCl extraction*

Determination	HL-A		
	2	7	5
ID_{50}	0.03	0.04	2.25
AD_{50}	8.5×10^2	10^3	6×10^4
CE_{50}	2.2×10^3	3×10^3	1.7×10^5
SR[a]	75	56	
Recovery	31%	36%	

[a] Specificity ratio.

The serological equivalent of the immunogenic yield, the absorptive yield, has been defined as the ratio of the number of intact cells required to absorb half of the cytotoxic activity of the alloantibody (AD_{50}) to the ID_{50} (in cell equivalents). The 3 M KCl extract had an absorptive yield (AD_{50}/ID_{50}) of 31% of the HL-A 2 antigen and 36% of the HL-A 7 antigen present on the intact cell surface [Table 5; (104)].

Although quantitative serological parameters were defined in this system, the microdroplet technique is a difficult and tedious one when thousands of reaction droplets are required to describe significantly the potency of a single antigen. Techniques employing radioactive labels for soluble antigen and for alloantibody are expected to increase the sensitivity and flexibility of the serological assay system. These methods are not subject to the vagaries of the complement-dependent cytotoxic system and do not require a panel of target cell donors. Quantitative serological analysis of the behavior of soluble antigens has afforded criteria for assessing the degree of purification, for comparing the potency of alloantigens prepared by a variety of techniques, and for determining the binding affinity of various alloantisera detecting a single specificity.

CHEMICAL CHARACTERIZATION OF SONICALLY TREATED TRANSPLANTATION ANTIGENS

Electrophoretically homogeneous guinea pig and human histocompatibility antigens extracted by sonic treatment have distinct, characteristic, reproducible chemical properties. Since only extremely few guinea pigs were available and because of the limitations of preparative PAGE systems, purified antigen could only be isolated in microgram quantities. The 0.73 to 0.74 guinea pig component was electrophoretically homogeneous upon reelectrophoresis in the presence of 8 M urea and in gels of varying porosity. Reelectrophoresis of ^{125}I-labeled purified component showed only a single sharply resolved electrophoretic component (Fig. 16A). Interestingly, strain 2 and strain 13 guinea pig transplantation antigens had the same electrophoretic mobility; admixtures

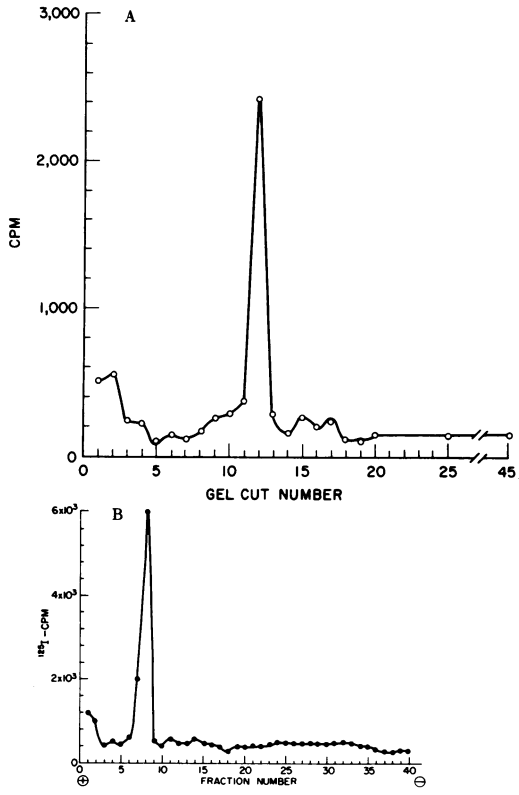

FIG. 16. *Electrophoretic homogeneity of purified sonically treated antigens: reelectrophoresis of the active antigenic component R_F 0.73 to 0.74 which had been radiolabeled with ^{125}I to increase the sensitivity in detecting microheterogeneity. Part A shows one sharp peak R_F 0.73 in the guinea pig preparation, suggesting that the material was electrophoretically homogeneous at pH 9.4 (62). Part B shows a sharp peak with R_F 0.80 in the human cell line RPMI 1788 (101).*

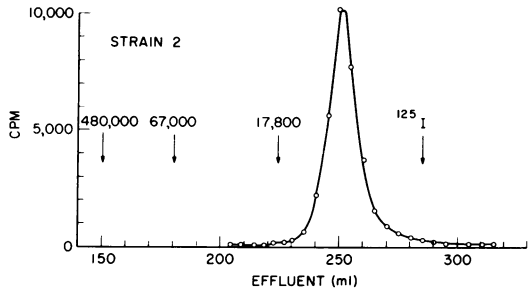

FIG. 17. *Molecular weight by calibrated gel filtration. Purified guinea pig antigen was passed through a Sephadex G-100 column equilibrated with 5 M guanidine. The K_D of elution was consistent with a molecular weight of 15,000.*

of the two antigens yielded a single component upon relectrophoresis (57).

Three independent techniques were employed to determine the molecular weight of guinea pig transplantation antigen to be about 15,000, assuming a partial specific volume of 0.74: (i) ultracentrifugal analyses using the Yphantis sedimentation equilibrium method at different speeds and monitored with interference optics (62), (ii) gel filtration on calibrated columns in the presence and absence of 5 M guanidine hydrochloride (Fig. 17), and (iii) calculation from the integral amino acid composition (n = 1, about 15,000). The three methods suggested size homogeneity in addition to electrophoretic homogeneity.

The amino acid compositions of the antigens obtained from the two strains of histoincompatible guinea pigs were characteristic and reproducible. They had statistically significant differences in their content of serine, leucine, isoleucine, alanine, and valine, and possibly also tyrosine and phenylalanine (Table 6). The differences ranged from 1 to 7.5 moles per cent. Hexosamine and half-cysteine residues were not detectable, and methionine was present in only trace amounts. The remaining amino acids were present in very similar quantities in both antigens (58).

These guinea pig transplantation antigens, which bear allotypic differences detected by biological tests reflecting transplantation individuality, probably express their antigenic markers in amino acid differences. Several investigators have demonstrated a correlation between allotypic specificities and the amino acid sequence of immunoglobulin heavy and light polypeptide chains (71, 93). Thus, at least some of the amino acid differences reflect critical sequences affecting conformation and antigenic expression.

The amino acid differences had additional significance since there was no detectable lipid or carbohydrate in the antigen. Folch partition

TABLE 6. *Amino acid compositions of allogeneic guinea pig transplantation antigens*[a]

Amino acid[b]	Strain 2[c]	Strain 13[b]	Difference[d] (strain 2 − strain 13)
Lysine	7.94 ± 0.44	8.25 ± 0.81	−0.31
Histidine	2.23 ± 0.18	2.34 ± 0.50	−0.11
Arginine	3.64 ± 0.28	2.96 ± 0/20	+0.68
Aspartic acid	11.84 ± 0.30	11.98 ± 0.49	−0.14
Threonine	6.42 ± 0.02	6.06 ± 0.07	+0.36
Serine[d]	10.95 ± 0.18	18.87 ± 0.04	−7.92
Glutamic acid	16.63 ± 0.71	16.75 ± 0.21	−0.12
Proline	4.30 ± 0.26	4.29 ± 0.81	+0.01
Alanine[d]	11.40 ± 0.08	7.96 ± 0.15	+3.44
Half-cysteine	Absent	Absent	0.00
Valine[d]	6.46 ± 0.13	5.48 ± 0.20	+0.98
Methionine	0.74 ± 0.22	Trace	+0.74
Isoleucine[d]	4.46 ± 0.16	3.32 ± 0.01	+1.14
Leucine[d]	8.58 ± 0.05	7.19 ± 0.03	+1.39
Tyrosine	0.86 ± 0.16	1.66 ± 0.24	−0.80
Phenylalanine	3.74 ± 0.27	2.96 ± 0.13	+0.78
Hexosamine	Absent	Absent	0.00

[a] Values are expressed as moles per cent.

[b] Glycine not determined due to residual binding after dialysis.

[c] Mole per cent of each amino acid with standard deviations calculated from two analyses.

[d] Difference of means in mole per cent: $2 < 13$ is negative, $2 > 13$ is positive.

[d] Amino acids which differed significantly at the P 0.01 level.

thin-layer chromatography on silica gel G was performed after lipid extraction with chloroform-methanol (2:1, v/v). Selective staining failed to reveal lipids, glycolipids, or cholesterol esters. The hexose and pentose content was less than the 1% threshold of cysteine-sulfuric acid method.

Chemical characterization of human histocompatibility antigens isolated from spleens was also limited by the microgram quantities of highly purified antigen available from a single donor (64). The active antigenic component isolated by PAGE possessed an R_F 0.80 at *p*H 9.4 (Fig. 16B). The molecular weight of HL-A alloantigen estimated by sedimentation equilibrium and by calculation from the integral amino acid composition was 34,600. The material showed size homogeneity; it was 94% monodisperse. The amino acid compositions of human histocompatibility antigens had general characteristics similar to those of guinea pigs: no detectable half cysteine or hexosamine and little methionine. Neither carbohydrate nor lipid moieties was detectable within the 1% limitation of the methods.

Amino acid analyses of purified antigens from cell lines RPMI 1788 and RPMI 4098 showed differences in the content of aspartic acid, serine, proline, alanine, and tyrosine analogous to those found among guinea pig transplantation antigens. These data again strongly suggested that the genetically segregating alloantigenic specificities reflecting histocompatibility were expressed by protein structure.

Genetic theory is also consistent with the histocompatibility loci coding polypeptide chains rather than carbohydrate moieties, as seen in the ABO system (90). Sharp insertions, noted in the ABO system and thought to reflect the action of enzymes performing specific maneuvers on precursor molecules, are not characteristic of transplantation specificities, which frequently are broad (24), similar to those observed with proteins. Ceppellini et al. (17) argued persuasively that the great number of mutants characterizing the HL-A system suggest a similarity to immunoglobulin allotypes, wherein many mutants are distributed along a limited number of linked structural genes, corresponding to the many antigenic determinants of a few polypeptide chains rather than to the ABO system in which a limited number of antigenic variants are controlled by a few enzymes acting on a common substrate. The mutants can then be assigned to different structural genes and to different sites within those genes by extrapolation from the polypeptide to the nucleotide sequence through the principle of collinearity (93).

Conclusions regarding the biochemical genetics of transplantation antigens must await the elucidation of their chemical structure. One would predict regions of constant amino acid composition, probably related to the biological role of these materials in the cytoarchitecture or in membrane function. The constant regions would be phylogenetically homologous. By analogy with other allotypic systems, there would be regions of variability, such as the C-terminal region of the heavy chain described by Tosi et al. (121) and the 34 N-terminal residues reflecting the a_1 and a_3 heavy-chain determinants, which occur independent of compositional changes associated with antibody specificity (21, 70, 71, 128). With more abundant source materials and effective techniques for antigen solubilization and purification, relatively large amounts of homogeneous material are now available to elucidate the structure of the chemical markers of transplantation individuality, thereby clarifying the genetics of histocompatibility.

SUMMARY

Transplantation antigens can be identified by the specific and potent immune reactions which they induce in allogeneic hosts. Solubilized derivatives obtained by the controlled application of sonic energy can be obtained from a wide variety of cells. These materials possess biological proper-

ties which suggest that they represent the specific antigenic sites of the cell surface in soluble form. Depending upon the dose and route of administration, they induce accelerated rejection or prolong the survival of donor-type tissue grafts. They elicit delayed-type hypersensitivity responses in preimmunized hosts. They can stimulate the production of or block the in vitro action of specific cytotoxic antisera. The extracts can be fractionated by a number of techniques, but the most effective purification method is preparative polyacrylamide gel electrophoresis. The purified material is a protein without detectable carbohydrate or lipid moieties. There are reproducible amino acid differences among antigens derived from histoincompatible individuals, suggesting that the genes at certain histocompatibility loci directly code the synthesis of polypeptide chains. Further information on the structure of these antigens will provide insight into the chemical nature of the determinants of histocompatibility and aid in the understanding of the mechanisms of allograft destruction, immunosurveillance, growth regulation, and cell-surface structure and function.

LITERATURE CITED

1. Al-Askari, S., J. R. David, H. S. Lawrence, and L. Thomas. 1965. In vitro studies on homograft sensitivity. Nature 205:916–917.
2. Al-Askari, S., and H. A. Lawrence. 1969. In vitro expression of homograft reactions. Transpl. Proc. 1:400–402.
3. Alling, D., and B. D. Kahan. 1969. Growth rates of mixed leukocyte cultures. Transplantation 8:1–8.
4. Amos, D. B. 1970. Genetics of the HL-A system, transplantation antigen symposium, Fed. Proc. 29:2018–2025.
5. Aronson, J. D. 1933. Tissue culture studies of the relation of the tuberculin reaction to anaphylaxis and the Arthus phenomenon. J. Immunol. 25:1–9.
6. Arquilla, C. R., and J. Finn. 1963. Genetic differences in antibody production. Science 142:400–401.
7. Bailey, D. W. 1965. A search for genetic background influences on survival times of skin grafts from mice bearing Y-linked histocompatibility. Transplantation 3:531–534.
8. Batchelor, R., and N. Selwood. 1970. HL-A subloci: are they valid, p. 243–249. *In* Histocompatibility testing. Munksgaard, Copenhagen.
9. Bennett, B., and B. R. Bloom. 1968. Reactions in vivo and in vitro produced by a soluble substance associated with delayed-type hypersensitivity. Proc. Nat. Acad. Sci. U.S.A. 59:756–762.
10. Billingham, R. E., L. Brent, and P. B. Medawar. 1956. The antigenic stimulus in transplantation immunity. Nature 178:514–519.
11. Bloom, B. R., and B. Bennett. 1968. Migratory inhibitory factor associated with delayed-type hypersensitivity. Fed. Proc. 27:13–15.
12. Boyse, E. A., L. S. Old, and E. Stockert. 1968. Location of antigenic substances on cell membrane surfaces. Proc. Nat. Acad. Sci. U.S.A. 60:886–892.
13. Brent, L., J. B. Brown, and P. B. Medawar. 1962. Quantitative studies on tissue transplantation immunity. VI. Hypersensitivity reactions associated with the rejection of homografts. Proc. Roy. Soc. Med. B156:187–209.
14. Broder, S. W., P. R. Glade, and K. Hirschorn. 1970. Establishment of long-term lines from small aliquots of normal lymphocytes. Blood 35:539–542.
15. Burch, P. R. J., and R. G. Burwell. 1965. Self and not self—a clonal induction approach to immunology. Quart. Rev. Biol. 40:252–279.
16. Burnet, M. 1970. Immunological surveillance. Sydney.
17. Ceppellini, R., E. S. Curtoni, P. L. Mattiuz, V. Miggiano, G. Scudeller, and A. Serra. 1967. Genetics of leukocyte antigens: a family study of segregation and linkage, p. 149–185. *In* Histocompatibility testing. Munksgaard, Copenhagen.
18. Choi, K. W., and A. D. Bloom. 1970. Cloning human lymphocytes in vitro. Nature 227:171–173.
19. Chrambach, A., and D. Rodbach. 1970. Polyacrylamide gel electrophoresis. Science, *in press*.
20. Clark, C., and J. Munoz. 1970. Delayed hypersensitivity to insulin and its component polypeptide chains. J. Immunol. 105:574–583.
21. Cohen, S., and C. Milstein. 1967. Structure and biologic properties of immunoglobulins. Advan. Immunol. 7:1–89.
22. Counce, S., P. Smith, R. Barth, and G. D. Snell. 1956. Strong and weak histocompatibility gene differences in mice. Ann. Surg. 144:198–204.
23. Crittenden, L. B., W. E. Briles, and H. A. Stone. 1970. Susceptibility to an avian leukosis-sarcoma virus: close association with an erythrocyte isoantigen. Science 169:1324–1325.
24. Dausset, J., J. Colombani, L. Legrand, and M. Fellons. 1970. Genetics of the HL-A system: deduction of 480 haplotypes, p. 53–75. *In* Histocompatibility testing. Munksgaard, Copenhagen.
25. David, J. R. 1966. Delayed hypersensitivity in vitro: its mediation by cell-free substances formed by lymphoid cell-antigen interactions. Proc. Nat. Acad. Sci. U.S.A. 56:72–77.
26. Davis, W. C., and L. Silverman. 1968. Localization of mouse H-2 histocompatibility antigen with ferritin-labelled antibody. Transplantation 6:535–543.
27. Dekaris, D., R. Fauve, and M. Raynaud. 1969. Delayed hypersensitivity and inhibition of macrophage spreading: in vivo and in vitro studies of tuberculin and streptococcal hypersensitivity in guinea pigs. J. Immunol. 103:1–5.
28. Diener, E., and W. D. Armstrong. 1967. Induction of antibody formation and tolerance in vitro to a purified protein antigen. Lancet ii:1281–1285.
29. Dutton, R. W., and R. I. Mishell. 1967. Cellular events in the immune response: the in vitro response of normal spleen cells to erythrocyte antigens. Cold Spring Harbor Symp. Quant. Biol. 12:407–441.
30. Edelman, G. M., B. A. Cunningham, W. E. Gall, P. D. Gottlieb, V. Tutishauser, and M. J. Waxdal. 1969. The covalent structure of an entire γG immunoglobulin molecule. Proc. Nat. Acad. Sci. U.S.A. 63:78–85.
31. Eichwald, E. J., and I. L. Weissman. 1966. Weak histocompatibility loci. Ann. N.Y. Acad. Sci. 129:94–101.
32. Ferguson, K. A. 1964. Starch gel electrophoresis: application to the classification of proteins and polypeptides. Metabolism 13:985–1002.
33. Fink, M. A., and V. A. Quinn. 1953. Antibody production in inbred strains of mice. J. Immunol. 70:61–67.
34. Fox, A. S. 1958. Genetics of tissue specificity. Ann. N.Y. Acad. Sci. 73:611–634.
35. Gasser, D. L. 1969. Genetic control of the immune response in mice. J. Immunol. 103:66–70.
36. George, M., and J. H. Vaughn. 1962. In vitro cell migration as a model for delayed hypersensitivity. Proc. Soc. Exp. Biol. Med. 11:514–521.
37. Globerson, A., and R. Auerbach. 1966. Primary antibody response in organ cultures. J. Exp. Med. 124:1001–1016.
38. Godfrey, H. P., H. Baer, and S. D. Chaparas. 1969. Inhibition of macrophage migration by a skin reactive polysaccharide from BCG culture filtrates. J. Immunol. 102:1466–1473.

39. Graff, R. J., and A. A. Kandutsch. 1966. Immunogenic properties of purified antigen preparation from a mouse sarcoma. Transplantation 4:465–473.
40. Graff, R., D. L. Mann, and S. G. Nathenson. 1970. Immunogenic properties of papain-solubilized H-2 alloantigens. Transplantation 10:59–65.
41. Graff, R. J., W. K. Silvers, R. E. Billingham, W. H. Hildemann, and G. D. Snell. 1966. The cumulative effect of histocompatibility antigens. Transplantation 4:605–617.
42. Granger, G. A., and W. P. Kolb. 1968. Lymphocyte in vitro cytotoxicity: mechanisms of immune and non-immune small lymphocyte-mediated target L cell destruction. J. Immunol. 101:111–120.
43. Haenen-Severyns, A., G. Degrovanni, A. Castermans, and G. Legeune. 1967. Improved method for preparing soluble transplantation antigen from normal mouse tissues, p. 289–293. *In* Advances in transplantation. Munksgaard, Copenhagen.
44. Hayey, P., and V. Defendi. 1970. Mixed lymphocyte cultures produce effector cells: model in vitro for allograft rejection. Science 168:133–135.
45. Heise, E. R., and R. S. Weiser. 1969. Factors in delayed sensitivity: lymphocyte and macrophage cytotoxins in the tuberculin reaction. J. Immunol. 103:570–576.
46. Hildemann, W. H., and N. Cohen. 1967. Weak histocompatibilities: emerging immunogenetic roles and generalizations, p. 13–20. *In* Histocompatibility testing. Munksgaard, Copenhagen.
47. Hilgert, I. 1967. A qualitative difference between certain H-2 and non-H-2 antigens responsible for a similar graft rejection rate. Experimentia 23:857–858.
48. Hirata, A. A., and P. Terasaki. 1970. Cross-reactions between streptococcal M proteins and human transplantation antigens. Science 168:1095–1096.
49. Jerne, N. 1970. Relation of histocompatibility, tolerance and antibody production. *In* Immunosurveillance mechanisms. (Brook Lodge Conference), Academic Press Inc., *in press*.
50. Jirgenson, B., and G. F. Springer. 1968. Conformation of blood group and virus receptor glycoproteins from red cells and secretions. Science 162:365–367.
51. Jones, A. L. 1966. The effect of polymorphonuclear leukocytes on the blastoid transformation of lymphocytes in missed leukocyte cultures. Transplantation 4:337–343.
52. Jones, J., E. Paraskova-Tchernozemska, and J. F. Moorhead. 1970. In vitro inhibition of DNA synthesis in human leukemic cells by a lymphoid cell extract. Lancet 1:654–655.
53. Kahan, B. D. 1965. Isolation of a soluble transplantation antigen. Proc. Nat. Acad. Sci. U.S.A. 53:153–161.
54. Kahan, B. D. 1965. A new method of bandaging orthoptic murine skin grafts. Proc. Soc. Exp. Biol. Med. 119:520–522.
55. Kahan, B. D. 1967. Cutaneous hypersensitivity reactions of guinea pigs to proteinaceous transplantation antigens. J. Immunol. 99:1121–1127.
56. Kahan, B. D., E. C. Holmes, R. A. Reisfeld, and D. Morton. 1969. Water-soluble guinea pig transplantation antigens from carcinogen induced sarcomas. J. Immunol. 102:28–36.
57. Kahan, B. D., and R. A. Reisfeld. 1967. Electrophoretic purification of a water-soluble guinea pig transplantation antigen. Proc. Nat. Acad. Sci. U.S.A. 58:1430–1437.
58. Kahan, B. D., and R. A. Reisfeld. 1968. Differences in the amino acid compositions of allogenic guinea pig transplantation antigens. J. Immunol. 101:237–241.
59. Kahan, B. D., and R. A. Reisfeld. 1968. Progress in the purification of a water-soluble guinea pig transplantation antigen, p. 295–299. *In* Advances in transplantation. Munksgaard, Copenhagen.
60. Kahan, B. D., and R. A. Reisfeld. 1969. Immunogenicity of electreophoretically purified guinea pig transplantation antigen. Proc. Soc. Exp. Biol. Med. 130:765–769.
61. Kahan, B. D., and R. A. Reisfeld. 1969. Transplantation antigens. Science 164:514–521.
62. Kahan, B. D., and R. A. Reisfeld. 1969. Advances in the chemistry of transplantation antigens. Transpl. Proc. 1:483–488.
63. Kahan, B. D., R. A. Reisfeld, L. B. Epstein, and J. G. Southworth. 1967. Biologic activities of water-soluble guinea pig transplantation antigen, p. 285–299. *In* Histocompatibility testing. Munksgaard, Copenhagen.
64. Kahan, B. D., R. A. Reisfeld, M. A. Pellegrino, E. S. Curtoni, P. L. Mattiuz, and R. Ceppellini. 1968. Water-soluble human transplantation antigens. Proc. Nat. Acad. Sci. U.S.A. 61:897–904.
65. Kahan, B. D., R. Zajtchuk, D. Dawson, and W. E. Adams. 1964. Kinetics of sensitization with whole and sonicated mouse spleen cells. Dis. Chest 46:452–456.
66. Kandutsch, A. A., and J. H. Stimpfling. 1965. Properties of purified isoantigenic preparation from a mouse sarcoma, p. 134–144. *In* Immunopathology, Congress VI. Karger and Sons, Geneva.
67. Kasakura, S., and L. Lowenstein. 1965. A factor stimulating DNA synthesis derived from the medium of leucocyte cultures. Nature 208:794–795.
68. Klein, J. 1967. Strength of histocompatibility genes in mice, p. 21–27. *In* Histocompatibility testing. Munksgaard, Copenhagen.
69. Koldovsky, P., A. Turano, and G. Fadda. 1969. Attempts to produce in vitro the reaction of immunologically competent cells of C57BL/6 origin and Balb-c embryo fibroblasts. J. Cell Physiol. 74:31–36.
70. Koshland, M. E. 1967. Location of specificity and allotypic amino acid residues in antibody Fd fragments. Cold Spring Harbor Symp Quant. Biol. 32:119–127.
71. Koshland, M. E., R. A. Reisfeld, and S. Dray. 1968. Differences in amino acid composition related to allotypic and antibody specificity of rabbit IgG heavy chains. Immunochemistry 5:471–483.
72. Kosnunen, T. V., B. H. Waksman, M. F. Flax, and W. S. Tihen. 1963. Radioautographic study of cellular mechanisms in delayed hypersensitivity. Immunology 6:276–290.
73. Lawrence, H. S. 1965. Bacterial hypersensitivity as the basis of the allograft reaction, p. 141–150. *In* Histocompatibility testing, NAS-NRC Publication 1229.
74. Lawrence, H. S., and A. M. Pappenheimer. 1957. Effect of specific antigen on release from human leukocytes of the factor concerned in the transfer of delayed hypersensitivity. J. Clin. Invest. 36:908–909.
75. Lilly, F., E. A. Boyse, and L. J. Old. 1964. Genetic basis of susceptibility to viral leukemogenesis. Lancet ii:1207–1209.
76. Lowenstein, L. 1967. Discussion comment, p. 302. *In* Histocompatibility testing. Munksgaard, Copenhagen.
77. McDevitt, H. O., and M. Sela. 1965. Genetic control of the antibody response. J. Exp. Med. 122:517–531.
78. McDevitt, H., and A. Chinitz. 1969. Genetic control of the antibody response: relationship between immune response and histocompatibility H-2 type. Science 163:1207–1208.
79. McKhann, C. F. 1964. Additive effective of multiple weak incompatibilities in transplantation immunity. Nature 201:937–938.
80. McKhann, C. F. 1964. Transplantation studies of strong and weak histocompatibility barriers in mice. II. Tolerance. Transplantation 2:620–626.
81. Manson, L. A., G. V. Faschi, and J. Palm. 1963. An association of transplantation antigens with microsomal lipoproteins of normal and malignant tissues. J. Cell. Comp. Physiol. 61:109–118.
82. Manson, L. A., and T. Simmons. 1969. Induction of the alloimmune response in mouse lymphocytes by cell-free transplantation antigens in vitro. Transpl. Proc. 1:498–501.
83. Marbrook, J. 1967. Primary immune response in cultures of spleen cells. Lancet ii:1279–1281.
84. Martin, C. M., J. B. Waite, and N. B. McCullough. 1957. Antibody protein synthesis by lymph node homotransplants to a hypogammaglobulinemic adult. J. Clin. Invest. 36:405–421.

85. Mason, S., and N. L. Warner. 1970. The immunoglobulin nature of the antigen recognition site on cells mediating transplantation immunity and delayed hypersensitivity. J. Immunol. 104:762–765.
86. Medawar, P. B. 1944. Behavior and fate of skin homografts and skin autografts in rabbits. J. Anat. 78:176–190.
87. Medawar, P. B. 1963. The use of antigenic extracts to weaken the immunologic reaction against skin homografts in mice. Transplantation 1:21–38.
88. Merrill, J. P., E. A. Friedman, R. E. Wilson, and D. C. Marshall. 1961. The production of "delayed type" cutaneous hypersensitivity to human donor leukocytes as a result of the rejection of skin homografts. J. Clin. Invest. 40: 631–635.
89. Mills, J. A. 1966. The immunologic significance of antigen induced lymphocyte transformation in vitro. J. Immunol. 97:239–247.
90. Mitchison, N. A. 1968. Antigens. *In* Haldane and modern biology, p. 63–72. Johns Hopkins Press, Baltimore.
91. Moore, G. E., R. F. Gerner, and H. A. Franklin. 1967. Culture of normal human lyukocytes. J. Amer. Med. Ass. 199:519–524.
92. Nakano, M., and W. Braun. 1967. Cell-released non-specific stimulators of antibody forming spleen cell populations. J. Immunol. 99:570–575.
93. Natvig, J. B., H. G. Kunkel, and S. P. Litwin. 1967. Genetic markers of the heavy chain subgroups of human G globulin. Cold Spring Harbor Symp. Quant. Biol. 32:173–180.
94. Oppenheim, J. J., B. G. Leventhal, and E. M. Hersh. 1968. The transformation of column purified lymphocytes with nonspecific and specific antigenic stimuli. J. Immunol. 101: 262–270.
95. Papermaster, V. M., B. W. Papermaster, and G. E. Moore. Histocompatibility antigens of human lymphocytes in long-term culture. Fed. Proc. 28:379.
96. Pellegrino, M. A., A. Pellegrino, and B. D. Kahan. 1970. HL-A antigens of human fetal cells. Transplantation 10: 425–430.
97. Rapaport, F. T., and R. M. Chase. 1965. The bacterial induction of homograft sensitivity. J. Exp. Med. 122:733–744.
98. Rapaport, F. T., J. Dausset, J. M. Converse, and H. S. Lawrence. 1965. Biologic and ultrastructural studies of leucocyte fractions as transplantation antigens in man. Transplantation 3:490–500.
99. Reif, A. 1966. An experimental test of two general relationships to describe the adsorption of antibodies by cells and tissues. Immunochemistry 3:267–268.
100. Reisfeld, R. A., and B. D. Kahan. 1970. Transplantation antigens. Advan. Immunol. 12:117–190.
101. Reisfeld, R. A., M. Pellegrino, B. W. Papermaster, and B. D. Kahan. 1970. Biologic and chemical characterization of human transplantation antigen. Fed. Proc., *in press*.
102. Reisfeld, R. A., M. Pellegrino, B. W. Papermaster, and B. D. Kahan. 1970. HL-A antigen from a continuous lymphoid cell line derived from a normal donor. J. Immunol. 104: 560–565.
103. Reisfeld, R. A., M. A. Pellegrino, B. W. Papermaster, and B. D. Kahan. 1970. Serologic characterization of soluble HL-A antigens from continuous lymphoid cell lines derived from normal donors, p. 445–460. *In* Histocompatibility testing. Munksgaard, Copenhagen.
104. Reisfeld, R. A., M. Pellegrino, B. W. Papermaster, and B. D. Kahan. 1970. Human transplantation antigens. *In* Proc. VI. Int. Cong. Immunopath., *in press*.
105. Rich, A. R., and M. R. Lewis. 1932. The nature of allergy and tuberculosis as revealed by tissue culture studies. Johns Hopkins Med. J. 50:115–131.
106. Rocklin, R. E., O. L. Meyers, and J. R. David. 1970. An in vitro assay for cellular hypersensitivity in man. J. Immunol. 104:95–102.
107. Rosenau, W., and H. D. Moon. 1961. Lysis of homologous cells by sensitized lymphocytes in tissue culture. J. Nat. Cancer Inst. 27:471–477.
108. Ruddle, N. H., and B. H. Waksman. 1968. Cytotoxicity mediated by soluble antigen and lymphocytes in delayed hypersensitivity. J. Exp. Med. 128:1237–1254.
109. Salvin, S. B., and J. Nishi. 1969. In vitro cell reactions in delayed hypersensitivity. J. Immunol. 103:138–141.
110. Schecter, G. P., and W. McFarland. 1970. Interaction of lymphocytes and a radioresistant cell in PPD stimulated human leukocyte cultures. J. Immunol. 105:661–669.
111. Seborg, M. 1967. In vitro detection of cellular hypersensitivity in man. Acta Med. Scand. 182:167–174.
112. Shimada, A., and S. G. Nathenson. 1969. Murine histocompatibility H-2 alloantigens. Biochemistry 8:4048–4062.
113. Snell, G. D. 1968. The H-2 locus of the mouse: observations and speculations concerning its comparative genetics and polymorphism. Folia Biol. 14:335–358.
114. Spitler, L. E., and H. H. Fudenberg. 1970. Products of interaction of antigen-sensitized leucocytes and antigen: further characterization of the mitogenic factor. J. Immunol. 104:544–549.
115. Springer, G. F., H. G. Schwick, and M. A. Fletcher. 1969. The relationship of the influenza virus inhibitory activity of glycoproteins to their molecular weight and sialic acid content. Proc. Nat. Acad. Sci. U.S.A. 64:634–641.
116. Tao, T. W., and J. W. Uhr. 1966. Primary type antibody response in vitro. Science 151:1096–1098.
117. Tennant, J. R., and G. D. Snell. 1968. H-2 locus and viral leukomogenesis as studies in congenic strains of mice. J. Nat. Cancer Inst. 41:597–604.
118. Terasaki, P. J., and J. D. McClelland. 1964. Microdroplet assay of human serum cytotoxins. Nature 204:998–1000.
119. Thomas, L. 1959. Mechanisms involved in tissue damage by endotoxins of gram-negative bacteria, p. 451–468. *In* Cellular and humoral aspects of the hypersensitive state. John Wiley & Sons, Inc., London.
120. Thor, D., R. E. Jureziz, S. R. Veach, E. Miller, and S. Dray. 1968. Cell migration inhibition factor released by antigen from human peripheral lymphocytes. Nature 219:755–757.
121. Tosi, S., R. Mage, and S. Dubiski. 1970. Distribution of allotypic specificities A1, A2, A14, A15 among immunoglobulin G molecules. J. Immunol. 104:641–647.
122. Turk, J. L. 1967. Delayed hypersensitivity. John Wiley & Sons, London.
123. Turk, J. L., and J. Oort. 1963. A histologic study of the early stages of the development of the tuberculin reaction after passive transfer of cells labelled with ^{3}H-thymidine. Immunology 6:140–147.
124. Twomey, J. J., O. Sharkey, J. A. Brown, A. H. Laughter, and P. H. Jordan. 1970. Cellular requirements for the mitotic response in allogeneic mixed leukocyte cultures. J. Immunol. 104:845–853.
125. Valentine, F. T., and H. S. Lawrence. 1969. Lymphocyte stimulation: transfer of cellular hypersensitivity to antigen in vitro. Science 165:1014–1016.
126. Vaz, N. M., and B. B. Levine. 1970. Immune responses of inbred mice to repeated low doses of antigen: relationship to histocompatibility H-2 type. Science 168:852–854.
127. Ward, P. A., H. G. Remold, and J. R. David. 1969. Leukotactic factor produced by sensitized lymphocytes. Science 163:1079–1081.
128. Wilkinson, J. M. 1969. Variation in the N-terminal sequence of heavy chains of immunoglobulin G from rabbits of different allotypes. Biochem. J. 112:173–185.
129. Wilson, R. E. 1971. Prolongation of survival with cell free antigens. *In* Transplantation antigens: markers of biologic individuality. Academic Press Inc., New York.
130. Wilson, R. E., A. Rippin, C. R. Hayes, R. K. Dagher, and C. G. Busch. 1969. Prolonged renal allograft survival associated with antigen pretreatment. Transpl. Proc. 1:290–292.
131. Zajtchuk, R., B. D. Kahan, and W. E. Adams. 1966. Tissue distribution of water-soluble transplantation antigen. Dis. Chest 50:368–371.
132. Zweimann, B., and S. M. Phillips. 1970. In vitro lymphocyte reactivity during depression of tuberculin hypersensitivity with 6-mercaptopurine. Science 169:284–285.